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EVALUATION OF THE MILITARY EFFECTIVENESS OF CHLOR-FLOC WATER PURIFICATION TABLETS FOR TREATMENT OF WATERBORNE MICRO-ORGANISMS

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Tests were conducted to determine the efficacy of CHLOR-FLOC water purification tablets for treating microbiological contaminants in drinking water. The test waters represented various physical/chemical challenge conditions. Microbial challenges consisted of enteric bacteria (Klebsiella terrigena), enterovirus (Echovirus 1), protozoan oocysts (Cryptosporidium parvum), and cyst simulant (latex beads). Studies used the U.S. Environmental Protection Agency's interim "Guide Standard and Protocol for Testing Microbiological Water Purifiers" for guidance. Results indicated that the CHLOR-FLOC system effectively reduced both bacterial and viral challenge components of the study to the required levels under all conditions, but failed to physically remove the oocysts and cyst simulant to the required levels under all conditions. Viability studies also indicated that the chemical disinfection component of the CHLOR-FLOC tablets had minimal capabilities to kill the encysted organisms over the 20-minute contact time. The overall study results revealed that the CHLOR-FLOC system was not adequate to physically remove, or to provide adequate chemical disinfection of, Cryptosporidium oocysts to the required level of 99.9 percent reduction.

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PREFACE

The U.S. Army is interested in alternative drinking water disinfectants for treatments of individual soldier water supplies in the field as a replacement for the current iodine (Globaline) tablets. The use of iodine tablets is adversely impacted by reduced disinfection efficiency at low temperature and pH, especially for the enteroviruses, and at high pH for certain protozoan cysts such as Giardia and Cryptosporidium. Alsc, because of limitations in disinfection effectiveness, newer military doctrine requires two iodine tablets per liter of drinking water with a contact time of 35 minutes before human consumption. The use of two tablets contributes significant adverse organoleptic (taste and odor) properties to the treated water. These problems and the potential avoidance of water can be a serious concern where personnel may be required to consume up to 15 liters a day to prevent dehydration (especially in hot, arid climates). A new commercially available disinfectant-flocculating agent, CHLOR-FLOC, may provide improved water purification over a broad range of pH and temperature, and may not suffer from interferences by organic chemical demand in field water supplies.

SUMMARY

The U.S. Army is interested in innovative methods for the microbiological purification of drinking water from highly contaminated sources. This study evaluates the efficacy of CHLOR-FLOC tablets for treating microbiological contaminants (bacteria, enteroviruses, and protozoan cysts) utilizing the U.S. Environmental Protection Agency's (USEPA) interim "Guide Standard and Protocol for Testing Microbiological Water Purifiers" as guidance in testing.

The tests, to determine the efficacy of CHLOR-FLOC for water purification, examined various physical/chemical challenge conditions, incorporating temperatures of 5 and 10 °C, pH of 4.5, 7.0, and 9.0, and general water qualities imparted by distilled-deionized halogen demand free, tap, and complex synthetic waters. The microbial challenges consisted of Klebsiella terrigena bacteria, Echovirus 1, Cryptosporidium parvum oocysts, and latex beads (a protozoan cyst simulant).

CHLOR-FLOC purification of challenge waters was provided for a period of 20 minutes, using two tablets for the 5 °C studies and one tablet for the 10 °C experiments. At the end of the disinfection-flocculation period, the samples were filtered through flannelette bags, treated with sodium thiosulfate to neutralize the chlorine, and then assayed for surviving micro-organisms. Suggested minimal microbial removal requirements to be considered effective for military use are: 6 logs for enteric bacteria; 4 logs for enteric viruses; and 3 logs for protozoan cysts (or simulant) over the prescribed contact period.

The study demonstrated that the USEPA guide standard was appropriate for use in determining the effectiveness of the CHLOR-FLOC disinfectant for the removal of typical waterborne microbial indicators and pathogens at low temperatures. The results indicated that both bacterial and viral challenge components of the study were effectively reduced to the required levels under all conditions. This study also showed that physical removals (coagulation and filtration) of the protozoan oocysts and latex beads (cysts simulant) were essentially equivalent; however, their physical removal alone was not adequate to meet the military removal requirements. Experiments examining the effectiveness of CHLOR-FLOC's disinfection component for viability of Cryptosporidium oocysts showed that the disinfectant had very minimal capabilities to kill the encysted organisms over the 20-minute contact time.

INTRODUCTION

The U.S. Army Biomedical Research and Development Laboratory (USABRDL) conducted low temperature efficacy studies on the disinfectant tablet CHLOR-FLOC, for the disinfection and physical removal of typical waterborne enteric microorganisms (bacteria, virus, and protozoan cysts), to determine if CHLOR-FLOC meets military requirements for individual soldier water purification. CHLOR-FLOC contains a disinfectant, sodium dichloro-s-triazinetrione dihydrate, at a concentration of 2.5 percent; and it also contains a complex coagulant mixture to clarify the water of turbidity, larger micro-organisms, and some organic chemicals such as humic and fulvic acids. The components of this complex coagulating mixture are: aluminum sulfate, sodium carbonate, bentonite, sodium carboxy-methylcellulose, Syloid-244, and Superfloc (a polyacrylamide). After coagulation, an important step in the CHLOR-FLOC treatment process is final filtration through a tightly-woven flannelette filter bag, after the disinfection process is complete, to remove flocculated materials which may contain some residual microbiological population.

While CHLOR-FLOC has been approved by the U.S. Environmental Protection Agency (USEPA)[1] especially for recreational and emergency uses at higher water temperatures (e.g., 20 °C), few studies have been conducted at low temperatures. No studies have been conducted under the USEPA's new interim "Guide Standard and Protocol for Testing Microbiological Water Purifiers"[2] (hereafter referred to as the guide standard and protocol), which, in the future, may be mandated for the marketing of point of use microbiological water purifiers in the United States. Because the military requires microbiological purification of waters of wide diversity and quality at all temperatures, the following study was performed to assess CHLOR-FLOC in typical-use scenarios. The study conditions imposed may not fully account for the very worst quality waters which the individual may be required to treat and consume on a worldwide basis, but should provide a representative challenge for most of the microbiological contaminant levels expected in the more typical water resources.

Collaborative research efforts between the USABRDL and the University of Arizona (UA), Department of Veterinary Science, Tucson, AZ, were also conducted to determine the relative effectiveness of CHLOR-FLOC and Army issue Globaline (iodine) tablets for disinfection of Cryptosporidium parvum oocysts, utilizing animal infectivity as the measure of effectiveness. These studies were necessary when it became apparent from USABRDL in-house efforts that the oocysts were not effectively removed physically by the CHLOR-FLOC filtration component of the treatment process. Studies were conducted in two phases: phase I - evaluation of the viability of oocysts solely by disinfection

using CHLOR-FLOC (without filtration) with comparative tests using Globaline tablets for disinfection; phase II - evaluation of the residual infectivity of oocysts after disinfection and subsequent filtration through flannelette material using CHLOR-FLOC. [The flannelette bag material was provided by the U.S. Army Natick Research, Development and Engineering Center (a prototype material developed by their laboratory).] All CHLOR-FLOC operational and testing procedures were identical to those used during the USABRDL in-house tests except that the strain of Cryptosporidium oocysts used was that provided by the UA.

USABRDL IN-HOUSE STUDIES MATERIALS AND METHODS

1. MICROBIOLOGICAL CHALLENGE

The CHLOR-FLOC disinfection studies utilized the USEPA's guide standard and protocol for testing guidance. However, the selection of the microbiological challenges was somewhat different than those indicated in that document. Table 1 shows the waterborne microbiological challenges and their removal endpoint requirements. It should be noted that the challenge for viruses is limited to a single virus, Echovirus 1, and excluded rotavirus (which was necessary because of limitations in the time and availability of the appropriate cell cultures). Also, the Giardia sp. cyst challenge was replaced in the test protocol by Cryptosporidium parvum oocysts because it was felt that the Cryptosporidium represented a worse challenge to the CHLOR-FLOC by their smaller size and supposed resistance to common water disinfectants. Also, military exposures to Cryptosporidium are thought to be of worldwide significance.

TABLE 1. MICROBIOLOGICAL CHALLENGES AND REMOVAL ENDPOINTS

Test Organisms	Challenge Levels/Liter	Minimum Removal in Log $_{10}$ (%)
Bacteria - Klebsiella terrigena (overnight culture)	10^8 CFU	6 (99.9999)
Enteric virus - Echovirus 1	10^7 PFU	4 (99.99)
Protozoan cysts - Cryptosporidium parvum	10^6 cysts	3 (99.9)
Latex bead cyst simulant - 3.7 μm AccuBeads™	10^7 beads	3 (99.9)

a. Klebsiella terrigena bacterium preparation

Klebsiella terrigena (#33257) was obtained from the American Type Culture Collection (Rockville, MD), grown in nutrient broth, and frozen at -70 °C in 1.0 ml volumes for the testing seed stock. To prepare seed inoculum for each test day, Klebsiella was grown overnight in nutrient broth and centrifuged at 8000 rpm for 10 minutes using a Sorvall GSA rotor. The pelleted bacteria were resuspended and washed three times in demand-free phosphate buffered saline (DFPBS), and then filtered through a Whatman # 2 filter pad to remove bacterial clumps. The filtered Klebsiella cells were diluted in DFPBS and adjusted to a scale reading of 35 using a Klett-Summerson colorimeter. A 1 ml volume of this suspension was added to each liter of test water to provide a challenge of approximately 1.0 X 10^8 CFU/L.

b. Echovirus 1 test preparation, purification and separation

Echovirus 1, V239 strain, (obtained from Dr. Mark Sobsey, University of North Carolina) was used to prepare the challenge virus. A 150 cm^2 monolayer of confluent BGMK cells (Whittaker Bioproducts, Walkersville, MD) was inoculated with 0.5 ml of stock seed virus at a multiplicity of infection (MOI) of 10 plaque forming units per cell (PFU/cell). After incubation for 1 hour at 36 °C, 50 ml of Earles Minimum Essential Medium (MEM) containing 2 percent fetal calf serum (FCS) was added to the infected cells. When total cytopathic effect (CPE) with minimal cell detachment was observed, the liquid was collected and centrifuged at 10,000 X g for 30 minutes. The supernatant fluid was discarded, and the pelleted cells were saved. Twenty ml of DFPBS, pH 7.2, was added to the flask; and the attached cells were removed from the surface with a cell scraper and combined with the pelleted cells from above. The BGMK cell suspension was "freeze-thawed" three times in an ethanol-dry ice bath to release the virus and then extracted three times in a Waring blender for 1 minute with 1,1,2-trichlorotrifluoroethane at a ratio of 4 ml per 6 ml of virus suspension. After each extraction, layers of the mixture were separated by centrifugation at 800 X g for 10 minutes in a refrigerated Sorvall centrifuge, and the aqueous phases were collected and pooled. The aqueous volume containing the virus was concentrated to a final volume of 10 ml with an Amicon Centriprep™ concentrator (30,000 MW cutoff) by centrifugation at 1500 X g in an IEC refrigerated centrifuge. The virus particles were separated by rate-zonal centrifugation,[3] using 10 to 30 percent sucrose gradients prepared in phosphate buffered demand-free water (0.05 M PO_4 buffer, pH 7.2), at 90,000 g in a Beckman SW28 rotor for 2 hours and 15 minutes. The gradient was collected in 2 ml fractions and assayed on BGMK cells by the plaque assay titration method. Fractions F-9 through F-14 and F-20 were combined to give a proportional number of small, medium and large aggregates. This combination yielded 3.15 X 10^9 PFU/ml when assayed on BGMK cells.

c. Cryptosporidium parvum oocyst preparation

Calf feces (50 percent in 2.5 percent potassium dichromate) containing Cryptosporidium parvum oocysts was obtained from the University of Idaho, Dept. of Veterinary Science, Caldwell, ID, and purified using the method of E. Waldman et al.[4] The calf feces suspension was dispensed into 50 ml polypropylene conical centrifuge tubes in 10 ml volumes, and an equal volume of pH 7.0 PBS containing 0.1 percent Tween 20 was added to each tube. The contents in the tubes were thoroughly mixed and centrifuged at 750 X g for 15 minutes. The liquid portions were discarded, and the pellets were resuspended in 15 ml PBS with Tween 20. Anhydrous ether (5.0 ml) was added and mixed with each of the suspensions for 1 minute. The tubes were then centrifuged at 500

X g for 10 minutes. The top three layers (ether, debris plug and PBS with Tween 20) were removed and discarded. The pelleted cysts were resuspended in 10 ml of PBS and combined; then they were centrifuged again. A small volume of the liquid was retained in the tube to resuspend the pelleted cysts. Cysts were further purified by the Percoll™ (Sigma # P-1644) discontinuous density gradient method. The Percoll™ was diluted in 0.15 M NaCl to densities of 1.04 and 1.08. The gradients were prepared in 10 ml centrifuge tubes by layering 3 ml of the 1.04 density on top of 3 ml of the 1.08 density Percoll.™ A volume of 0.5 ml of the oocysts was layered on the surface of each gradient in the centrifuge tube, and the tubes were centrifuged at 250 X g for 10 minutes at room temperature. The upper bands, lower bands, and pellets were collected separately from the tubes, diluted in 10 volumes of PBS, and then centrifuged at 500 X g for 10 minutes. The PBS-Percoll™ fluids were discarded; then the pellets were resuspended in a small volume of PBS with 0.01 percent Tween 20 and examined microscopically for oocysts. The oocysts, which were concentrated in the resuspended pellet from the lower bands, were diluted in PBS with 0.01 percent Tween 20 to contain approximately 1.66 X 10^6 oocysts/ml. Oocysts were added to each test beaker to furnish 1.5-2.0 X 10^6/l.

d. Latex bead (protozoan simulant) preparation

A suspension of AccuBead™ particles (latex beads) with a mean diameter size of 3.7 μm (geometric standard deviation = 1.03), was obtained from FASTEK (A Kodak Company, Liverpool, NY). The latex beads were prepared in sterile deionized-distilled water (dd H_2O) containing 50 μg/ml of sodium dodecyl sulfate (added to reduce bead clumping caused by electrostatic attraction). The beads were added to the 1-liter test beakers of water to provide a final concentration of 1.5-2.0 X 10^7 beads/l.

2. PHYSICAL/CHEMICAL WATER CHARACTERISTICS AND TEST PROCEDURES

The tests to determine the effectiveness of CHLOR-FLOC examined a range of physical/chemical challenge conditions in various disinfectant free waters (Table 2).

TABLE 2. WATER QUALITY CHALLENGE CONDITIONS

Water	Temp(°C)	pH	Turbidity NTU	TDS mg/l	TOC mg/l
DF[1]	5 & 10	5.0, 7.0, 9.0	≤5.0	50-500	≤5.0
Tap[2]	5 & 10	5.0, 7.0, 9.0	≤5.0	50-500	≤5.0
WC[3]	5 & 10	5.0, 7.0, 9.0	≥30.0	≥1500	≥10.0

1 = distilled-deionized halogen-demand free water
2 = tapwater 3 = worst case water

a. Demand-free water, glassware and reagents

Halogen demand-free water (HDFW) was prepared by addition of 10 mg/l chlorine to dd H_2O. After storage in the dark at room temperature for 24 hours, the water was dechlorinated by exposure to sunlight. Buffer solutions used to adjust the pH of the test waters were prepared in HDFW. All glassware items for these tests were washed, cleaned in a solution of sulfuric acid containing No-Chromix,™ washed again in detergent, rinsed with distilled water, soaked overnight in a 20 mg/l chlorine solution, and rinsed in HDFW. The glassware was sterilized in a dry-heat oven at 150 °C for 90 minutes.

b. Test waters

Water characteristics, pH and temperature were adjusted for each run according to each set of conditions being tested. For each run, four beakers were filled with 1 liter of challenge water. All challenge organisms were added to three of the beakers (two for replicate CHLOR-FLOC disinfectant challenge and one control to determine viability of all challenge organisms over the test period); and the fourth beaker of water served as a control to measure CHLOR-FLOC disinfectant levels over the test period. Challenge organisms were added to provide the following approximated concentrations per liter of water: 1.0×10^8 Klebsiella terrigena cells; 2.0×10^6 Cryptosporidium parvum oocysts; 2.0×10^7 latex beads; and 1.0×10^8 PFU Echovirus 1.

(1) Tapwater

Tapwater was dechlorinated by continuous stirring at room temperature for 24-48 hours. Four beakers, each containing 1 liter of the dechlorinated water, were placed in a precooled circulator waterbath. After the water in each beaker reached the appropriate temperature, the pH was adjusted accordingly with 1.0 N NaOH or 1.0 N H_2SO_4.

(2) Worst case (WC) water

Tapwater was dechlorinated by continuous stirring at room temperature for 24-48 hours. After measuring 1 liter of the dechlorinated water into each of four beakers, the following components were added to each beaker: 1500 mg sea salt; 10 mg humic acid; 150 mg AC Test Dust to give a turbidity of 30 NTU. When the water reached the appropriate temperature, the pH was adjusted accordingly with NaOH or H_2SO_4 as above.

(3) Demand-free (DF) water

Four 1-liter beakers of HDFW were adjusted to the appropriate temperature and pH for each test with NaOH or H_2SO_4 as above.

c. Test procedures

Components of the CHLOR-FLOC individual water purification system used in these tests are shown in Figure 1. CHLOR-FLOC tablets were added to the test beakers according to the manufacturer's recommendations for treating natural resource waters (as represented in this study by DF, Tap, and WC waters). Two tablets were added to 5 °C water, and one tablet was added to 10 °C water. In all tests the CHLOR-FLOC tablets were added to 1 liter of the prescribed water and dissolved by stirring the water for 1 minute. The water components were allowed to settle for 4 minutes, then stirred vigorously for a few seconds, followed by a 15-minute interval for coagulation and flocculation. After a total contact time of 20 minutes, the entire sample was filtered through a flannelette bag provided by the manufacturer into a separate container. Figure 2 shows the flocculation and coagulation in tap and worst case 5 °C waters 5 minutes after treatment with two CHLOR-FLOC disinfectant tablets. Figure 3a shows the appearance of the final product tapwater compared with the 5-minute treated tapwater, while figure 3b compares the worst case final product water with the 5-minute treated worst case water. Microbiological samples were taken from the reaction mixture just before CHLOR-FLOC addition, at 5 minutes upon redispersing the floc after the initial mixing/settling period, and after filtration through the flannelette material (which provided a total reaction time of 22 minutes). Samples were taken from the viability control beaker, which contained the prescribed water and organisms without the CHLOR-FLOC, at 0 time and after filtration through the flannelette bags at 22 minutes; and similarly from the disinfectant control beaker, which contained only the CHLOR-FLOC in the water to measure residual disinfectant. In all cases the test samples were immediately neutralized with sodium thiosulfate except for the CHLOR-FLOC measurement control. After each use, the flannelette bags were backflushed with tapwater to remove trapped organisms and beads, immersed briefly in a mild detergent solution, and thoroughly rinsed in deionized distilled water. The bags were randomly used throughout the test runs and were replaced when they became thin or torn.

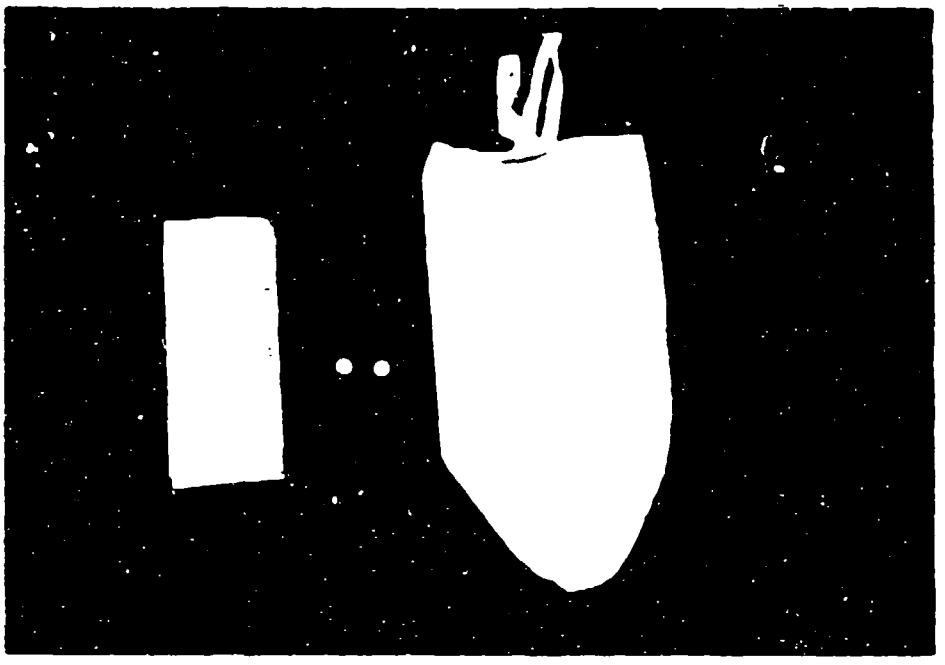


Figure 1. CHLOR-FLOC water purification system components

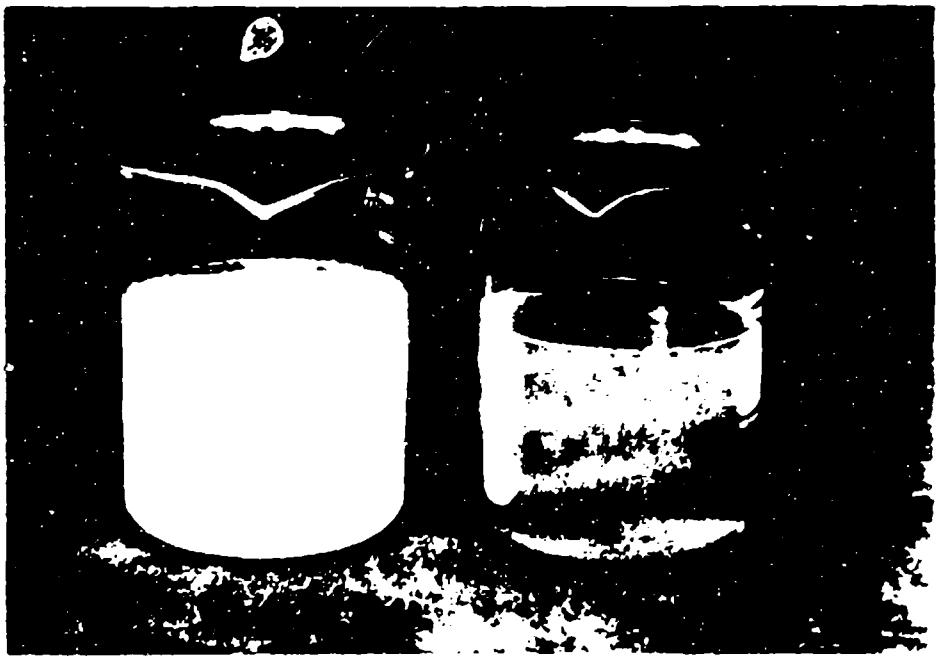


Figure 2. Flocculation/coagulation with two CHLOR-FLOC tablets in tapwater (left) and worst case water (right)

Figure 3. CHLOR-FLOC treated waters at 5 °C
a. Tapwater (before and after bag filtration)

Figure 3. CHLOR-FLOC treated waters at 5 °C
b. Worst case water (after and before bag filtration)

A modification to the above procedure was used to conduct additional experiments to determine if the maintenance of high test water pH would interfere with the coagulation, filtration, or disinfection of the test micro-organisms. This was considered necessary because the CHLOR-FLOC tablets are buffered to lower the pH of the water to less than pH 7.0. However, under typical conditions it is not unrealistic to expect that field drinking waters could be naturally buffered at higher pH which would overcome the low pH buffering capacity of the tablets. For this evaluation, test water pH values were adjusted and maintained at pH 9.0, using an inorganic base (KOH), after CHLOR-FLOC addition. The test organisms were added 1 minute after CHLOR-FLOC addition, when the waters had stabilized at pH 9.0, to insure that the initial high pH of the water (approaching pH 11.0) before CHLOR-FLOC addition was not deleterious to the test virus and bacteria.

3. SAMPLE ANALYSES

a. Chlorine analysis

Free available chlorine (FAC) was determined by the LaMotte-Palin DPD chlorine test procedure. Samples from each test water were collected for FAC level determinations 1 minute after addition of CHLOR-FLOC tablets to the challenge waters and immediately following filtration through the flannelette material after the 20 minute treatment period.

b. Bacteriological analysis

Sample analyses were performed using the m-Endo medium for the membrane filtration procedure as described in the USEPA guide standard and protocol. Ten ml samples of each test water were collected before CHLOR-FLOC was added; and after the disinfectant was added, samples were collected at 5 minutes and after flannelette bag filtration at 20 minutes. Samples were immediately placed in tubes which contained sodium thiosulfate to neutralize the chlorine. Serial 10-fold dilutions were made in PBS, and triplicate 1 ml volumes of each dilution were filtered through the 0.45 μm porosity Millipore bacteriological filters. Colonies were counted after 24 hours incubation at 35 °C.

c. Virus analysis

Test samples were taken from each beaker before CHLOR-FLOC was added; and after CHLOR-FLOC was added, samples were collected at 5 minutes and after the 20-minute treatment period following bag filtration. The 5 ml samples were immediately added to 5 ml of 2X MEM containing 2 percent newborn calf serum and 1 percent sodium thiosulfate. Subsequent dilutions were made in 1X MEM containing 2 percent newborn calf serum. A volume of 0.25 ml of each virus dilution was inoculated onto triplicate 72-hour BGMK

cell monolayers grown in 60 mm tissue culture dishes; the cells were then incubated in 5 percent CO_2 at 37 °C, rocked at 15-minute intervals for 1 hour, and overlaid with 5 ml of Medium 199 containing antibiotics (5 units/ml Nystatin, 0.05 mg/ml Gentamycin, and 5 units/ml Penicillin-Streptomycin), 2 percent newborn calf serum, 1.4 percent Difco purified agar, 0.125 percent sodium bicarbonate and 0.01 M Hepes buffer. Following incubation in 5 percent CO_2 for 48-56 hours at 37 °C, each plate was overlaid with 4 ml of Hanks BSS containing 5 percent neutral red stain. The stain was allowed to absorb for 1 hour and then poured off. Plaques were counted 12 hours after staining.

d. Protozoan cyst and cyst simulant analyses

Before adding the CHLOR-FLOC tablets, 100 ml samples were collected from each beaker of the untreated water which contained micro-organisms and latex beads. At the end of the CHLOR-FLOC treatment period the seeded waters were filtered through flannelette bags, and 500 ml samples were collected (approximately 22 minutes after disinfectant tablets were added). All samples were immediately dechlorinated with sodium thiosulfate. After addition of 0.1 percent Tween 20, each sample was separately filtered through a 47 mm Nuclepore 1.0 μm polycarbonate membrane filter to collect the oocysts and beads. The filter membrane was then cut into four sections and placed into a polypropylene centrifuge tube. Oocysts and beads were washed from the membrane filter surface by adding 10 ml of PBS (containing 0.01 percent Tween 20) to the tube and vigorously mixing the tube contents on vortex mixer. The membrane filter was washed five times. After each wash, the liquid portion, which contained the suspended oocysts and beads, was removed from the tube and combined in a new tube. The oocysts and beads, collected in the wash solutions, were concentrated by centrifugation at 1200 X g for 20 minutes. The liquid portion was removed and discarded except for approximately 1 ml which was used to resuspend the pellet of cysts and beads. The resuspended cysts and beads were stained with 0.5 percent Malachite Green for 20 minutes at room temperature and then decolorized with 0.25 percent sulfuric acid prior to quantitation with a hemacytometer using Nomarski differential interference contrast with a 63X objective. Background debris retained the green color, whereas the oocysts and beads appeared colorless.

UA COLLABORATIVE STUDIES MATERIALS AND METHODS

For the collaborative studies, the USABRDL provided all necessary personnel, supplies, equipment, and the CHLOR-FLOC water purification tablets; and USABRDL personnel performed the actual treatment procedures on the waters, including oocysts dosing, as described for in-house efforts. The studies were physically conducted at the UA Veterinary Department of Science Laboratories. The UA collaborators provided the Cryptosporidium oocysts, neonatal test animals, animal per diem, test animal holding facilities, and reagent waters used in the study. The UA veterinary staff also determined the appropriate cyst dosages for the infectivity studies, prepared the various disinfectant-treated test water samples for animal infectivity studies, prepared and infected the animals with control and treated cysts, and performed histological examinations of the gastrointestinal tract of the animals for evidence of Cryptosporidium infection.

1. MICROBIOLOGICAL CHALLENGE

Cryptosporidium parvum oocyst production and purification

Cryptosporidium oocysts were recovered from the feces of experimentally infected 2- to 5-day-old Holstein bull calves by a previously developed method.[5] Briefly, Cryptosporidium parvum infection was produced in calves by feeding $1.0\text{-}2.0 \times 10^8$ infective oocysts suspended in 1 liter of reconstituted commercial milk replacer. The calves were isolated in vealer pens; and the feces excreted during the peak oocyst shedding period were collected, mixed with an equal volume of 5 percent potassium dichromate ($K_2Cr_2O_7$), and stored at 4 °C. The collected feces were sieved sequentially through stainless steel screens of decreasing aperture ending with 63 μm pore size (230 mesh). Sequential discontinuous sucrose gradient (1.064 to 1.103 g/ml) centrifugation, followed by isopycnic Percoll™ gradient (1.091 g/ml) centrifugation, completed oocyst purification. The purified oocysts were stored in 2.5 percent potassium dichromate at 4 °C. Oocysts were withdrawn from storage as needed for experimentation and washed with PBS (0.025 M, pH 7.4) by filtration through polycarbonate filters (1-3 μm pore size) or by centrifugation to remove the dichromate storage solution.

2. PHYSICAL/CHEMICAL WATER CHARACTERISTICS AND TEST PROCEDURES

a. Test Waters

The designated 1-liter test waters were prepared as described in the USABRDL in-house studies above.

b. Phase I test procedures

USABRDL staff dosed the waters with the oocysts in accordance with the standard test procedure described earlier, except that the CHLOR-FLOC product was not filtered through flannelette material. CHLOR-FLOC or Globaline tablets were added at the prescribed levels--two Globaline tablets with a disinfection contact time of 35 minutes; and one CHLOR-FLOC tablet at 10 °C, or two CHLOR-FLOC tablets at 5 °C with a disinfection contact time of 20 minutes. The iodine and chlorine disinfectant levels were monitored in each sample to ensure that proper disinfection levels were attained and maintained throughout the appropriate disinfection contact time. The chlorine /iodine were rapidly neutralized with sodium thiosulfate. The samples were then handed over to the UA staff where the oocysts were concentrated from the treated water, physically enumerated, diluted for infectivity studies, and provided to the neonatal mice by gavage.

During phase I experiments, 1-liter volumes were collected from each of the Cryptosporidium seeded test waters (untreated, CHLOR-FLOC treated, and iodine treated). After the chlorine and iodine were neutralized, the samples were filtered through 1 μm pore size Nuclepore polycarbonate membrane filters. The filters were then carefully removed, placed in a 50 ml tube (tube A) with 10 ml of washing solution (1 liter of nanopure water containing 10 μl of Tween 20), and mixed with a vortex mixer for 15 seconds. The filter was removed and placed in another 50 ml tube (tube B) with an additional 10 ml of washing solution and mixed. The contents of tube B was added to tube A, and the filter in tube B was washed again. The filter in tube B was removed while the second wash from that tube was also combined with tube A. Tube B was washed twice with 7.5 ml of nanopure water and combined with tube A. The entire contents of tube A were centrifuged at 3000 rpm in a Sorvall T-6000B centrifuge with a H1000B rotor for 10 minutes. The liquid portion was aspirated down to 1 ml, and the pellet was thoroughly mixed with the ml of residual wash water. Appropriate dilutions of this concentrated sample were used to measure residual cyst concentrations and diluted for oral gavage of test animals.

c. Phase II test procedures

Testing procedures followed the same basic protocols as described above with the same division of responsibility. During these trials iodine disinfection was not evaluated. This research evaluated the capabilities of new prototype plastic reaction bags for floc formation and settling by the coagulating agents contained in the CHLOR-FLOC tablets. These bags have a spout on the bottom through which settled flocculated material can be wasted before the main bulk of water is filtered through flannelette. Figures 4a, b, and c depict the disinfection

process for a worst case water at 5 °C treated with two CHLOR-FLOC tablets as follows; (a) flocculation 5 minutes after treatment, (b) settled coagulated-flocculated material 20 minutes after treatment, and (c) filtration and collection of the product water after settled materials were discarded. The bags and also the flannelette material used in this effort were prototypes provided by the U.S. Army Natick Research, Development, and Engineering Center.

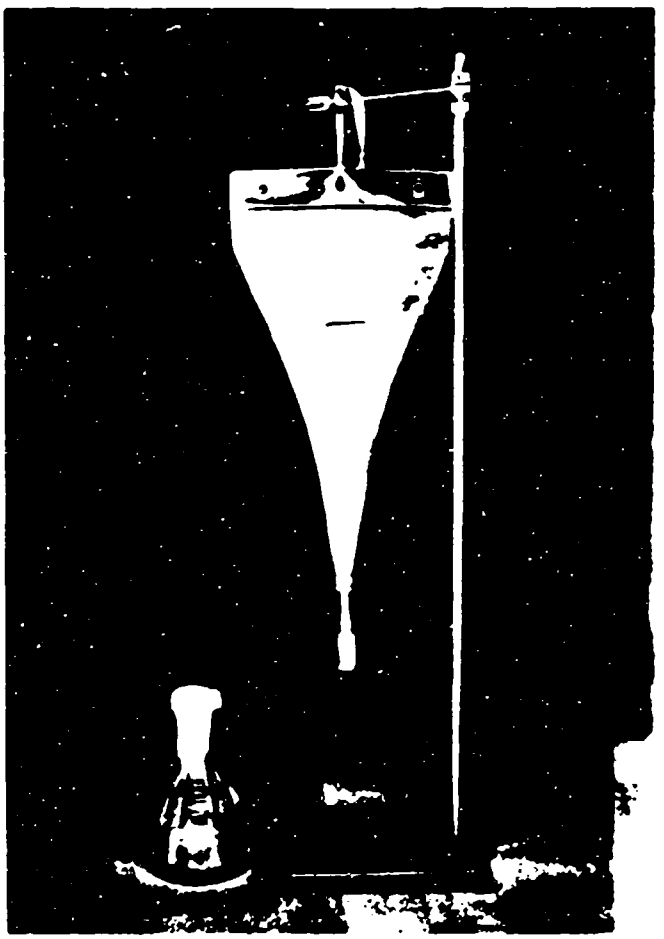


Figure 4. New CHLOR-FLOC system with worst case 5 °C water
a. Five minutes after treatment

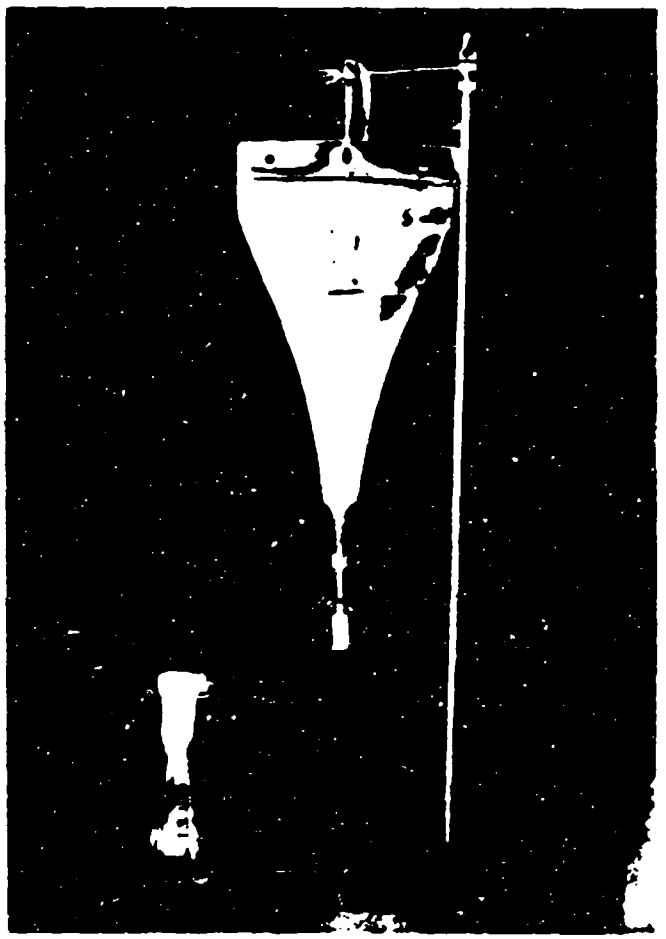


Figure 4b. Settled flocculated material 20 minutes after treatment

For phase II efforts a modified protocol for oocyst recovery from worst case water samples was utilized to circumvent problems observed with the recovery of cysts during phase I. The procedure was as follows: After neutralization of residual chlorine with sodium thiosulfate, the 1 liter samples were transferred equally into two 750 ml centrifuge bottles; the original bottles were washed with 10-20 ml of washing solution, and this material was added to the centrifuge bottles. The samples were centrifuged in a Sorvall T-6000B centrifuge with a T1000B rotor at 3500 rpm for 15 minutes. The supernatant fluid was aspirated to a few ml in the bottom of the bottles, and the pellet was resuspended into the residual fluid. The resuspended material was transferred into 50 ml tubes and centrifuged again at 3000 rpm for 10 minutes. The fluid was aspirated and the pellets were combined. Finally, the residual pellet was centrifuged as before and aspirated to 1.0 ml. This provided the material used for neonatal mouse dosing.

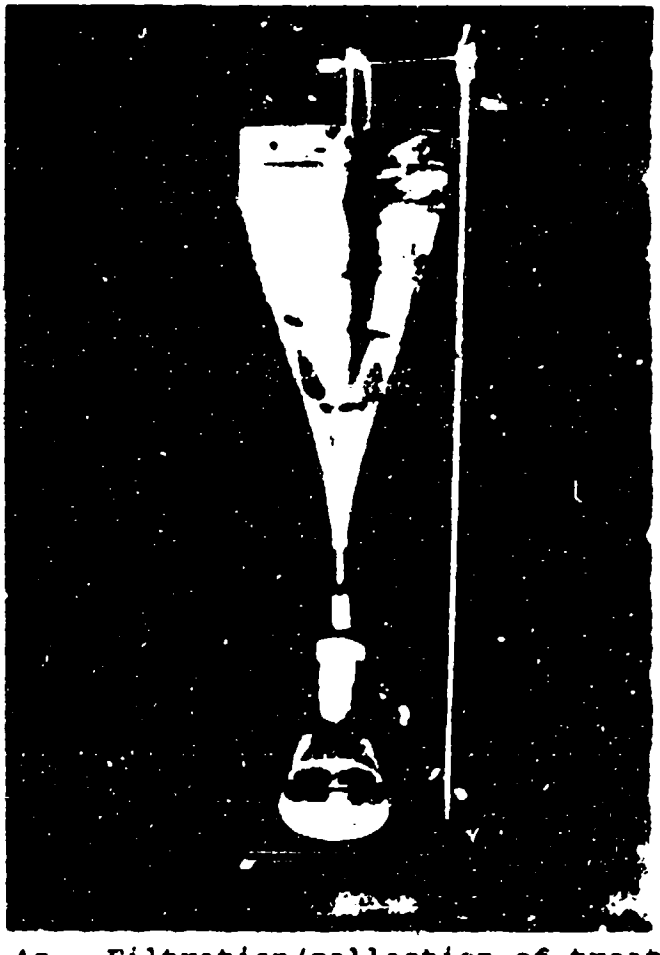


Figure 4c. Filtration/collection of treated water after settled materials were discarded

3. SAMPLE ANALYSES

Late-term pregnant female BALB/c mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). Within 24 hours of birth, mouse pups were randomized and placed back with the mothers (6 to 7 pups/litter) to minimize maternal effects on experimental outcomes. Animals were maintained in micro-isolate cages throughout the experiments. Room temperature was maintained at 18-26 °C with a 12-hour light/dark cycle and a relative humidity of 40-70 percent. Mice were fed Tekland Mouse/Rat Chow and sterile water ad libitum.

Previous experience with this mouse infectivity model indicated that neonatal mice can be routinely infected orally with 10^4 to 10^5 Cryptosporidium oocysts at 5 days of age. Gastrointestinal (GI) colonization and oocyst shedding develops

2-5 days later and resolves in about 5-7 days. BALB/c mice have been used for numerous experiments with Cryptosporidium.[6,7]. Sterling's laboratory has just completed a susceptibility dynamics infection in relation to the BALB/c neonatal mouse. They were able to infect 100 percent of the neonatal BALB/c mice through 9 days of age with 10^5 oocysts by oral intubation.

a. Phase I

Median infectious dose range determinations for the phase I study were conducted by infecting neonatal mice by gavage in which a range of Cryptosporidium oocysts were administered to groups of neonatal mice. The ID_{50} dose was determined by extrapolation from the dose range studies in which infection of intestinal villi was determined.

b. Phase II

During phase II studies the baseline ID_{50} was determined by an in vitro excystation method[8] (Appendix 1). The method involved triplicate determinations of excystation in which the relative numbers of intact oocysts, excysted shells, and sporozoites were counted; and the percent of theoretical sporozoite yield was produced. Then estimation of the ID_{50} from previously determined linear regression analysis of neonatal mouse infectivity vs. percent theoretical sporozoite yield was performed.

The protocol for experimental dosing utilized centrifuged sample preparations from 50 ml centrifuge tubes. The volume was increased to 5 ml (2 ml for centrifuged worst case water) with nanopure water, and the sample was mixed on a vortex mixer to uniformly disperse the oocysts. The dilutions needed to obtain the "high dose," "median dose," and "low dose" in 100 µl of inoculum, based on the previously determined ID_{50} and the recovery efficiencies were as follows:

# of oocyst seeded per liter X (recovery efficiency) divided by (ID_{50} X 10^5) = # of ml of dilution needed for 10,000 X ID_{50} in 100 µl. This was the "high dose."

Dilute the above suspension 1:10 to obtain 1,000 X ID_{50} in 100 µl. This was the "medium dose."

Dilute the "medium dose" suspension 1:10 to obtain 100 X ID_{50} in 100 µl. This was the "low dose."

For experimental studies, CHLOR-FLOC treated oocysts (ID_{50} dose and sequential 10-fold higher levels) were also administered by oral gavage to 5- to 7-day-old neonatal BALB/c mice. This was accomplished with a blunted slightly bent, 1/2 inch, 25-26 gauge hypodermic needle fitted with a short piece of polyethylene

tubing mounted on a 1 cc tuberculin syringe. The animals were sacrificed 7 days post inoculation; and approximately 3 cm of the terminal ileum was removed, fixed in 10 percent formalin, embedded in paraffin, and sectioned. Hematoxylin and eosin stained paraffin sections were examined microscopically for evidence of Cryptosporidium infection in the microvillous region of villous enterocytes. Specimens with parasitic stages present were scored as positive; those without were scored as negative. Positive specimens always showed numerous parasitic stages (at least 50-60 per 100X microscope field), while no parasites could be found on any sections taken from negative tissue samples. Infection was scored by the relative concentration of Cryptosporidium in the ileum ranging from a 0 to 4+ level of infection. Any level of infection was scored as a positive result.

During phase II, companion tests using fluorescent monoclonal antibody (OW 64 MAb) directed at the suture line of Cryptosporidium oocysts were utilized as a measure of infectivity for comparison against results from neonatal infectivity procedures. The suture line in oocysts appears only when the oocyst has experienced some degradation, and the antibody was prepared against this suture material. In this case the numbers of oocysts and their infectivity were determined microscopically using both a fluorescent monoclonal antibody for the cyst wall (total cysts) and one for the suture line. Because the antibodies were tagged with different color fluorescing dyes, the full cysts and cysts with suture lines could be discriminated and each could be counted.

1. USABRDL IN-HOUSE STUDIES

a. Free available chlorine

FAC levels produced by CHLOR-FLOC did not differ significantly between control and challenge waters or between halogen demand free, tap, and worst case waters. Nor was FAC affected significantly by pH, temperature or turbidity. The 1-minute readings from test waters ranged from 6-10 mg/l for a single CHLOR-FLOC tablet, and 20-minute sample readings ranged from 5-9 mg/l. It is assumed that two tablets would give proportionately the same results. The resuspended flocs interfered somewhat in visually determining color depth from the DPD tests and may have been the cause of the variable range in FAC levels for each tablet. However, all 20-minute samples including controls consistently showed a 1-2 mg/l decrease in FAC level when compared against their 1-minute level. The decreased levels of FAC could have been due to chlorine dissipation while the containers were uncovered during sampling, combination with water constituents, or impaired visual acuity from increased flocculation. If present, combined chlorine was not detected by increased color intensity using the DPD chlorine comparator.

b. Bacteria and virus removals

Disinfection/physical removal of Klebsiella terrigena in all waters at pH 7.0 exceeded the six-$\log_{10}$ removal requirements at both 5°C and 10°C (Tables 3 & 4). Tables 5 & 6 reveal that Echovirus 1 inactivation also exceeded the 4-$\log_{10}$ removal requirements for all waters and conditions at pH 7.0 for both temperatures. Both the Klebsiella terrigena and Echovirus 1 removals were attained within 5 minutes of contact with CHLOR-FLOC and did not require physical filtration of the flocculated mixture through the flannelette bag to achieve these removals. Similarly, the bacteria and virus removals from the various waters at pH of 4.5 and 9.0 were equivalent to the pH 7.0 results (e.g., no detectable organisms present at 5 minutes). These overall bacteria and virus removals (averaged from the initial pH 4.5, 7.0, and 9.0 waters) are shown in Figures 5 and 6. Not unexpectedly, the flannelette bag-filtered test samples taken at 22 minutes were also negative for the bacteria and viruses in all cases. The micro-organism control samples without CHLOR-FLOC, which received only filtration through the flannelette material after 20 minutes, experienced low, but variable, removals which indicate that the flannelette material had little effect in filtering out or adsorbing the bacteria and virus in the absence of CHLOR-FLOC. The reason for the variability seen in the controls in both the bacterial and viral removals was not determined. However, there are several

possibilities for the variability, such as initial aggregation of organisms in the unbuffered challenge waters which were dispersed during the sample dilutions in PBS, handling techniques by different operators, manufacturing differences in the flannelette material, or the number of times the filter bags were used.

As shown in duplicate tests, artificial maintenance of the pH at 9.0 in the various waters upon CHLOR-FLOC treatment did not interfere with the removal characteristics of either the Klebsiella terrigena bacteria or Echovirus 1 (Table 7); both were removed to the required levels within 5 minutes of contact, again without filtration. These averaged removals are also shown in Figures 5 and 6.

c. Cyst and cyst simulant removals

The removal of Cryptosporidium cysts (Tables 8 & 9) and the 3.7 μm AccuBeads™ (Tables 10 & 11) was determined only for physical removal as provided by coagulation and subsequent filtration through the flannelette material. In these experiments only pre-disinfection and 20-minute reaction mixtures (after filtration) were analyzed. For all three test waters at pH 7.0, two CHLOR-FLOC tablets at 5 °C achieved cyst removals ranging from 80.40 to 96.20 percent and latex bead removals ranging from 78.20 to 90.40 percent. Experiments conducted under identical conditions but at 10 °C with a single CHLOR-FLOC tablet produced similar results. Here, cyst removals ranged from 92.58 to 99.80 percent, while the cyst simulant removals ranged from 96.97 to 99.42 percent; only minor differences from this were noted in waters tested at pH 4.5 and 9.0. As seen in Figures 7 & 8, there was a general trend toward improved removals of both type particles as water quality decreased, i.e., worst case > tap > demand free distilled waters. Also, there was a tendency toward better removals of both cysts and beads at the higher temperature, possibly because of increased coagulation.

When pH 9.0 was maintained after CHLOR-FLOC addition, the cyst and simulant removals were slightly better (Table 12, Figures 7 & 8) compared against the ambient test pH results. Under these high pH conditions cyst removals ranged from 97.80 to >99.90 percent, and bead removals ranged from 93.49 to 99.71 percent. The comparison between the Cryptosporidium cyst and the 3.7 μm AccuBead™ removals indicates that the removals are very similar and that the beads are a credible simulant for evaluating the physical removal of these cysts. Beads could be used in future studies on large-scale batch water treatment with CHLOR-FLOC to determine Cryptosporidium cyst removals.

TABLE 3. *KLEBSIELLA TERRIGENA* DISINFECTION WITH TWO CHLOR-FLOC TABLETS AT 5 °C

Test Beaker #	Water Type	Sample Time (min)	Water pH 4.5 Inoculum CFU/L*	% Removal	Water pH 7.0 Inoculum CFU/L	% Removal	Water pH 9.0 Inoculum CFU/L	% Removal
1	Demand Free	0	1.23×10^8		8.87×10^7		1.17×10^8	
		5	0	>99.9999	0	>99.9999	0	>99.9999
2	"	0	1.33×10^8		9.10×10^7		1.23×10^8	
		5	0	>99.9999	0	>99.9999	0	>99.9999
Control	"	0	9.67×10^7		8.80×10^7		8.70×10^7	
		22	1.33×10^8	-37.54	8.77×10^7	0.34	1.10×10^8	-26.44
1	Tap	0	1.05×10^8		1.87×10^8		1.27×10^8	
		5	0	>99.9999	0	>99.9999	0	>99.9999
2	"	0	1.00×10^8		1.80×10^8		1.03×10^8	
		5	0	>99.9999	0	>99.9999	0	>99.9999
Control	"	0	1.27×10^8		1.43×10^8		1.37×10^8	
		22	1.07×10^8	15.75	1.60×10^8	-11.89	1.27×10^8	7.30
1	Worst Case	0	1.46×10^8		8.50×10^7		5.67×10^7	
		5	0	>99.9999	0	>99.9999	0	>99.9999
2	"	0	1.80×10^8		7.53×10^7		3.00×10^7	
		5	0	>99.9999	0	>99.9999	0	>99.9999
Control	"	0	1.27×10^8		7.57×10^7		3.67×10^7	
		22	1.34×10^8	-5.51	8.87×10^7	-17.17	9.00×10^7	-145.23

CFU/L: colony forming units/liter

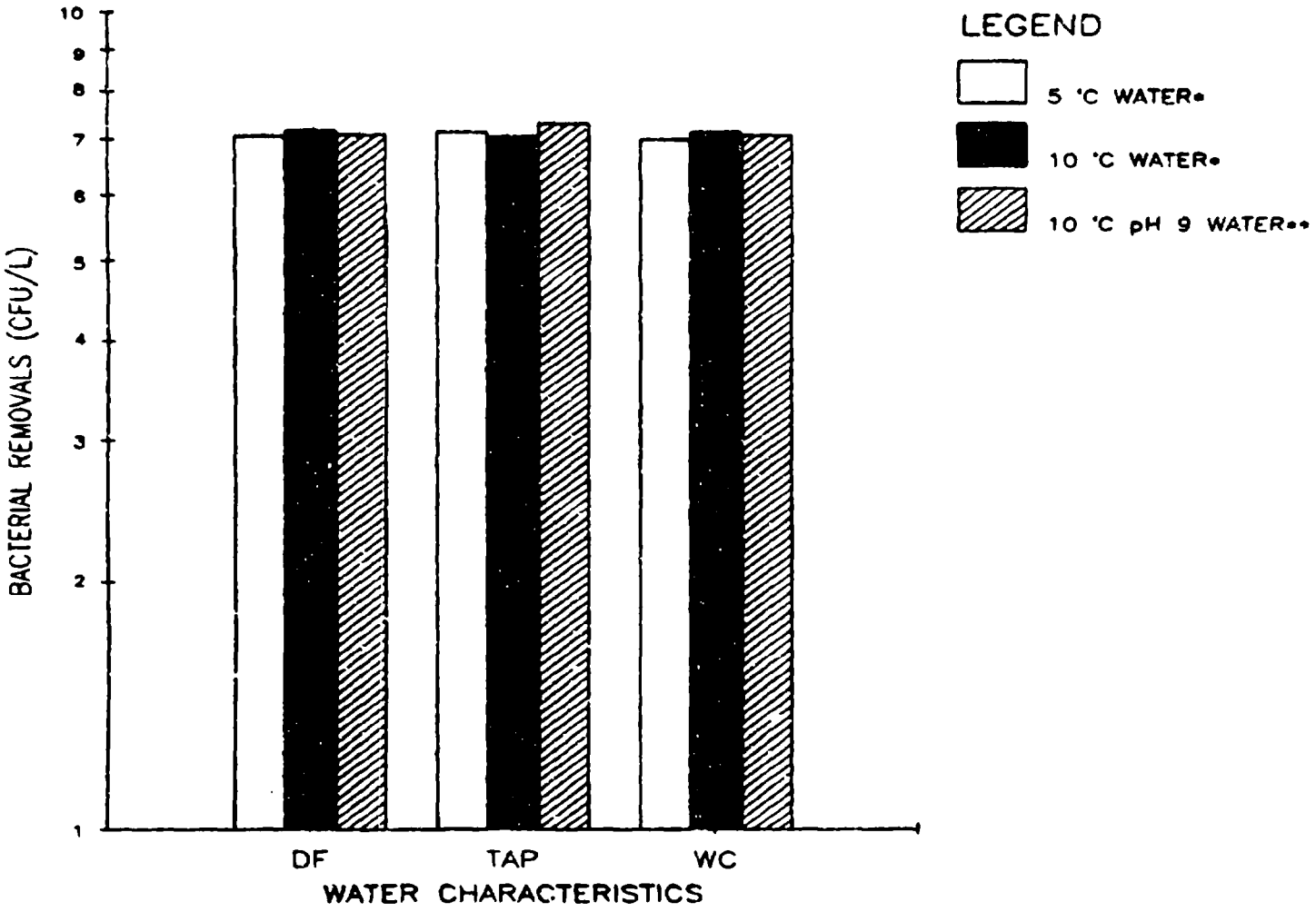
TABLE 4. *KLEBSIELLA TERRIGENA* DISINFECTION WITH ONE CHLOR-FLOC TABLET AT 10 °C

Test Beaker #	Water Type	Sample Time (min)	Water pH 4.5 Inoculum CFU/L*	% Removal	Water pH 7.0 Inoculum CFU/L	% Removal	Water pH 9.0 Inoculum CFU/L	% Removal
1	Demand Free	0	1.00×10^8		1.47×10^8		1.70×10^8	
		5	0	>99.9999	0	>99.9999	0	>99.9999
2	"	0	1.18×10^8		1.00×10^8		2.10×10^8	
		5	0	>99.9999	0	>99.9999	0	>99.9999
Control	"	0	1.37×10^8		1.13×10^8		1.90×10^8	
		22	9.07×10^7	33.80	1.08×10^8	4.42	1.43×10^8	24.74
1	Tap	0	8.33×10^7		1.20×10^8		1.17×10^8	
		5	0	>99.9999	0	>99.9999	0	>99.9999
2	"	0	1.00×10^8		1.20×10^8		1.10×10^8	
		5	0	>99.9999	0	>99.9999	0	>99.9999
Control	"	0	9.33×10^7		1.20×10^8		1.27×10^8	
		22	8.67×10^7	7.07	1.40×10^8	-16.67	3.00×10^7	76.38
1	Worst Case	0	1.47×10^8		1.10×10^8		1.10×10^8	
		5	0	>99.9999	0	>99.9999	0	>99.9999
2	"	0	1.57×10^8		1.33×10^8		1.33×10^8	
		5	0	>99.9999	0	>99.9999	0	>99.9999
Control	"	0	9.67×10^7		1.43×10^8		1.33×10^8	
		22	5.20×10^7	46.23	9.67×10^7	32.38	8.30×10^7	37.59

CFU/L*: colony forming units/liter

Figure 5.

KLEBSIELLA TERRIGENA DISINFECTION WITH CHLOR-FLOC



* Average of pH 4.5, 7.0, and 9.0 waters

** Average of duplicate tests

TABLE 5. ECHOVIRUS DISINFECTION WITH TWO CHLOR-FLOC TABLETS AT 5 °C

Test Beaker #	Water Type	Sample Time (min)	Water pH 4.5 Inoculum PFU/L*	% Removal	Water pH 7.0 Inoculum PFU/L	% Removal	Water pH 9.0 Inoculum PFU/L	% Removal
1	Demand Free	0	8.27×10^6		4.93×10^7		1.15×10^7	
		5	0	>99.99	0	>99.99	0	>99.99
2	"	0	6.27×10^6		4.40×10^7		1.23×10^7	
		5	0	>99.99	0	>99.99	0	>99.99
Control	"	0	8.40×10^6		4.93×10^7		1.48×10^7	
		22	8.67×10^6	-3.21	4.80×10^7	2.64	1.68×10^7	-13.51
1	Tap	0	6.40×10^7		7.47×10^7		4.53×10^7	
		5	0	>99.99	0	>99.99	0	>99.99
2	"	0	4.00×10^7		7.20×10^7		3.87×10^7	
		5	0	>99.99	0	>99.99	0	>99.99
Control	"	0	5.01×10^7		5.73×10^7		4.40×10^7	
		22	6.27×10^7	-25.15	6.40×10^7	-11.69	4.93×10^7	-12.05
1	Worst Case	0	3.20×10^7		1.89×10^7		2.21×10^7	
		5	0	>99.99	0	>99.99	0	>99.99
2	"	0	4.67×10^7		1.71×10^7		2.49×10^7	
		5	0	>99.99	0	>99.99	0	>99.99
Control	"	0	2.53×10^7		1.76×10^7		2.97×10^7	
		22	6.80×10^7	-168.77	1.76×10^7	0	1.92×10^7	35.35

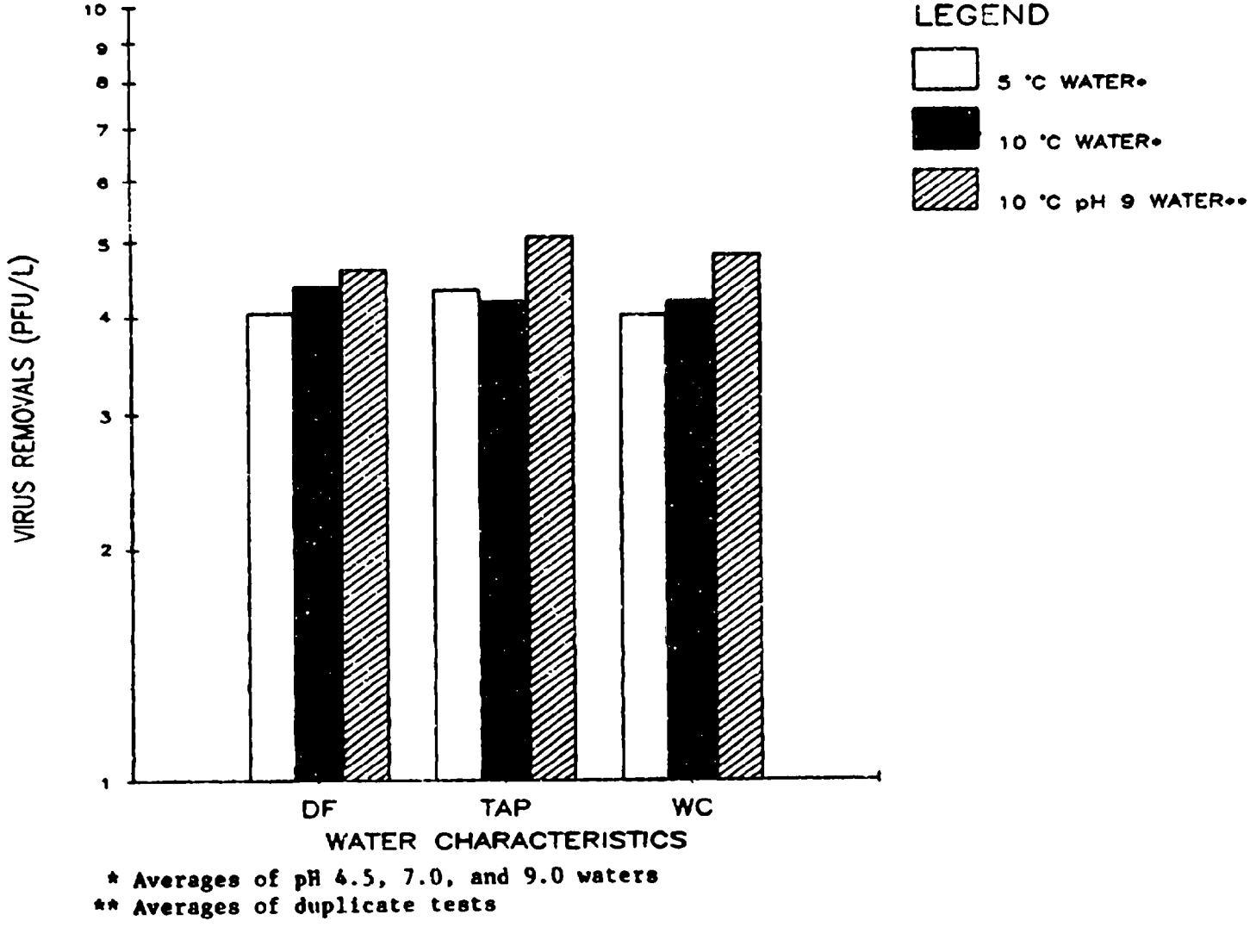
PFU/L: plaque forming units/liter

TABLE 6. ECHOVIRUS DISINFECTION WITH ONE CHLOR-FLOC TABLET AT 10 °C

Test Beaker #	Water Type	Sample Time (min)	Water pH 4.5 Inoculum PFU/L*	% Removal	Water pH 7.0 Inoculum PFU/L	% Removal	Water pH 9.0 Inoculum PFU/L	% Removal
1	Demand Free	0	2.67×10^7		2.57×10^7		1.32×10^8	
		5	0	>99.99	0	>99.99	0	>99.99
2	"	0	2.13×10^7		2.67×10^7		1.32×10^8	
		5	0	>99.99	0	>99.99	0	>99.99
Control	"	0	2.40×10^7		2.64×10^7		1.24×10^8	
		22	2.00×10^7	16.67	3.73×10^7	-41.29	1.29×10^8	-4.03
1	Tap	0	1.77×10^7		5.87×10^7		5.60×10^7	
		5	0	>99.99	0	>99.99	0	>99.99
2	"	0	2.02×10^7		4.53×10^7		4.13×10^7	
		5	0	>99.99	0	>99.99	0	>99.99
Control	"	0	2.12×10^7		5.47×10^7		4.93×10^7	
		22	1.97×10^7	7.08	4.00×10^7	26.87	3.60×10^7	26.98
1	Worst Case	0	3.24×10^7		2.28×10^7		5.07×10^7	
		5	0	>99.99	0	>99.99	0	>99.99
2	"	0	3.27×10^7		2.37×10^7		7.20×10^7	
		5	0	>99.99	0	>99.99	0	>99.99
Control	"	0	2.92×10^7		2.51×10^7		7.47×10^7	
		22	3.05×10^7	-4.45	3.07×10^7	-22.31	5.87×10^7	21.42

PFU/L*: plaque forming units/liter

Figure 6. **ECHOVIRUS DISINFECTION WITH CHLOR-FLOC**



* Averages of pH 4.5, 7.0, and 9.0 waters
** Averages of duplicate tests

TABLE 7. DISINFECTION OF *KLEBSIELLA TERRIGENA* AND ECHOVIRUS WITH ONE CHLOR-FLOC TABLET AT 10 °C WITH pH ADJUSTED TO MAINTAIN 9.0

Water Type	Sample #	Time	*Klebsiella terrigena* CFU*/L	% Reduction	Echovirus 1 PFU**/L	% Reduction
DF[1]	1	0	1.20×10^8		1.04×10^8	
		5	0	>99.9999	0	>99.99
DF	2	0	1.20×10^8		1.04×10^8	
		5	0	>99.9999	0	>99.99
DF	Control	0	1.03×10^8		9.73×10^7	
		22	8.30×10^7	19.42	7.72×10^7	20.66
Tap	1	0	1.97×10^8		3.00×10^8	
		5	0	>99.9999	0	>99.99
Tap	2	0	1.97×10^8		3.00×10^8	
		5	0	>99.9999	0	>99.99
Tap	Control	0	1.27×10^8		2.32×10^8	
		22	3.00×10^7	76.38	2.51×10^8	-8.19
WC[2]	1	0	1.13×10^8		1.60×10^8	
		5	0	>99.9999	5.33×10^3	>99.99
		22	-		0	>99.99
WC	2	0	1.13×10^8		1.60×10^8	
		5	0	>99.9999	1.07×10^4	>99.99
		22	-		0	>99.99
WC	Control	0	9.70×10^7		1.05×10^8	
		22	8.70×10^7	10.31	4.90×10^7	53.33

* CFU: colony forming units

** PFU: plaque forming units

DF[1]: demand free water

WC[2]: worst case water

TABLE 8. CRYPTOSPORIDIUM PARVUM OOCYST REMOVAL WITH TWO CHLOR-FLOC TABLETS AT 5 °C

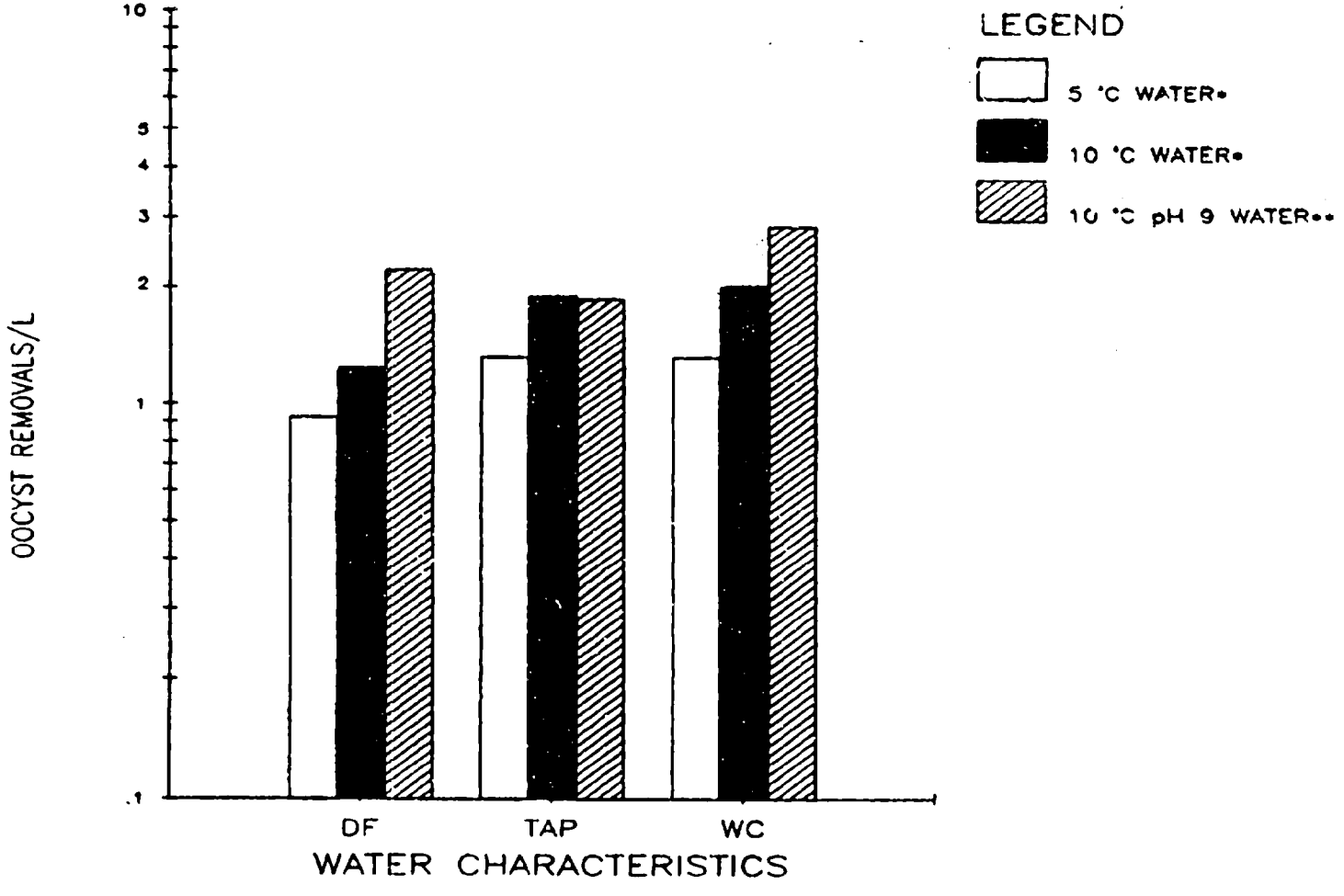
Test Beaker #	Water Type	Time (min)	Water pH 4.5 Oocysts/L	% Removal	Water pH 7.0 Oocysts/L	% Removal	Water pH 9.0 Oocysts/L	% Removal
1	Demand Free	0	1.93×10^6		1.68×10^6		1.94×10^6	
		22	1.60×10^5	91.71	2.76×10^5	83.60	1.60×10^5	91.76
2	"	0	1.59×10^6		1.77×10^6		2.04×10^6	
		22	2.16×10^5	86.42	3.47×10^5	80.40	1.50×10^5	92.65
Control	"	0	1.90×10^6		2.03×10^6		2.08×10^6	
		22	1.69×10^6	11.05	1.37×10^6	32.50	1.68×10^6	19.23
1	Tap	0	2.04×10^6		1.99×10^6		1.88×10^6	
		22	9.30×10^4	95.40	1.93×10^5	90.30	2.40×10^4	98.70
2	"	0	2.24×10^6		1.87×10^6		2.00×10^6	
		22	6.00×10^4	97.30	1.93×10^5	89.70	1.60×10^4	99.20
Control	"	0	1.72×10^6		1.97×10^6		1.92×10^6	
		22	1.42×10^6	17.40	1.78×10^6	9.60	1.54×10^6	20.00
1	Worst Case	0	1.60×10^6		1.14×10^6		1.76×10^6	
		22	1.27×10^5	92.10	1.18×10^5	89.60	6.40×10^4	96.36
2	"	0	1.64×10^6		1.73×10^6		1.88×10^6	
		22	6.12×10^4	96.30	6.60×10^4	96.20	4.00×10^4	97.87
Control	"	0	1.68×10^6		1.71×10^6		2.04×10^6	
		22	1.37×10^6	18.45	1.35×10^6	21.06	1.18×10^6	42.35

TABLE 9. CRYPTOSPORIDIUM PARVUM OOCYST REMOVAL WITH ONE CHLOR-FLOC TABLET AT 10 °C

Test Beaker #	Water Type	Time (min)	Water pH 4.5 Oocysts/L	% Removal	Water pH 7.0 Oocysts/L	% Removal	Water pH 9.0 Oocysts/L	% Removal
1	Demand Free	0	2.16×10^6		1.88×10^6		1.88×10^6	
		22	1.08×10^5	95.00	1.76×10^4	99.06	1.93×10^5	89.70
2	"	0	2.04×10^6		1.92×10^6		2.08×10^6	
		22	1.17×10^5	94.30	1.43×10^5	92.48	1.17×10^5	94.40
Control	"	0	2.16×10^6		1.96×10^6		2.12×10^6	
		22	1.97×10^6	8.80	1.82×10^6	7.14	1.70×10^6	17.00
1	Tap	0	2.09×10^6		2.00×10^6		1.80×10^6	
		22	1.52×10^4	99.30	3.60×10^4	98.20	3.80×10^3	99.79
2	"	0	1.84×10^6		1.96×10^6		2.00×10^6	
		22	3.37×10^4	98.20	4.54×10^4	97.68	2.09×10^4	98.95
Control	"	0	1.88×10^6		2.16×10^6		2.04×10^6	
		22	1.54×10^6	18.10	1.84×10^6	14.80	1.74×10^6	14.71
1	Worst Case	0	2.32×10^6		2.00×10^6		1.48×10^6	
		22	5.76×10^4	97.50	1.87×10^4	99.06	$<1.00 \times 10^3$	$\geq$99.90
2	"	0	1.94×10^6		1.84×10^6		1.60×10^6	
		22	3.17×10^4	98.40	3.60×10^3	99.80	$<1.00 \times 10^3$	$\geq$99.90
Control	"	0	2.11×10^6		1.92×10^6		1.63×10^6	
		22	1.54×10^6	27.00	9.92×10^5	48.00	8.00×10^5	50.90

Figure 7.

CRYPTOSPORIDIUM PARVUM OOCYST PHYSICAL REMOVALS WITH CHLOR-FLOC



* Averages of pH 4.5, 7.0, and 9.0 waters

** Averages of duplicate tests

TABLE 10. PROTOZOAN SIMULANT REMOVAL WITH TWO CHLOR-FLOC TABLETS AT 5 °C

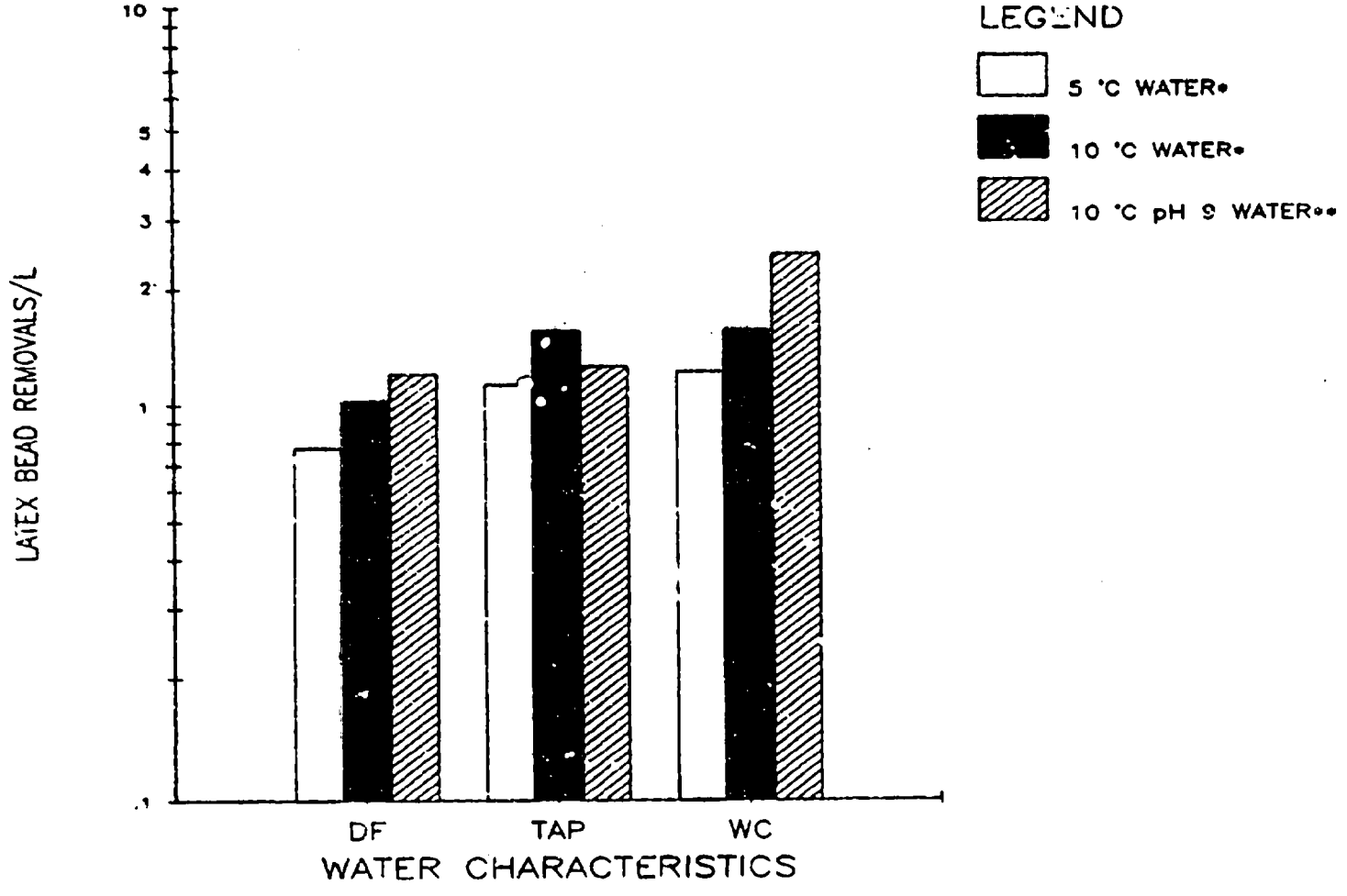
Test Beaker #	Water Type	Time (min)	Water pH 4.5 Accubeads/L	% Removal	Water pH 7.0 Accubeads/L	% Removal	Water pH 9.0 Accubeads/L	% Removal
1	Demand Free	0	2.23×10^7		1.11×10^7		2.12×10^7	
		22	4.26×10^6	80.90	2.39×10^6	78.47	2.21×10^6	89.58
2	"	0	1.82×10^7		1.37×10^7		2.16×10^7	
		22	3.99×10^6	78.08	2.99×10^6	78.20	2.42×10^6	88.80
Control	"	0	2.35×10^7		1.77×10^7		2.13×10^7	
		22	1.99×10^7	15.32	1.40×10^7	20.90	1.57×10^7	26.10
1	Tap	0	2.12×10^7		1.90×10^7		2.12×10^7	
		22	1.76×10^6	91.70	2.12×10^6	88.80	5.04×10^5	97.60
2	"	0	2.18×10^7		1.79×10^7		1.80×10^7	
		22	1.72×10^6	92.10	2.17×10^6	87.80	6.80×10^5	96.20
Control	"	0	1.71×10^7		1.81×10^7		1.95×10^7	
		22	1.48×10^7	13.40	1.47×10^7	18.80	1.44×10^7	26.15
1	Worst Case	0	1.86×10^7		1.02×10^7		1.28×10^7	
		22	1.34×10^6	92.80	1.13×10^6	88.90	9.68×10^5	92.44
2	"	0	2.06×10^7		1.32×10^7		1.34×10^7	
		22	1.01×10^6	95.10	7.98×10^5	94.00	2.40×10^5	98.21
Control	"	0	1.87×10^7		1.01×10^7		1.61×10^7	
		22	1.45×10^7	22.46	1.02×10^7	-0.99	7.20×10^6	55.28

TABLE 11. PROTOZOAN SIMULANT REMOVAL WITH ONE CHLOR-FLOC TABLE™ AT 10 °C

Test Beaker #	Water Type	Time (min)	Water pH 4.5 Accubeads/L	% Removal	Water pH 7.0 Accubeads/L	% Removal	Water pH 9.0 Accubeads/L	% Removal
1	Demand Free	0	1.98×10^7		1.90×10^7		1.80×10^7	
		22	3.16×10^6	84.00	3.84×10^5	98.00	2.48×10^6	86.20
2	"	0	2.63×10^7		1.53×10^7		2.00×10^7	
		22	2.39×10^6	90.91	4.64×10^5	96.97	2.45×10^6	87.80
Control	"	0	1.68×10^7		1.72×10^7		1.82×10^7	
		22	1.58×10^7	6.00	1.33×10^7	22.70	1.03×10^7	43.40
1	Tap	0	1.89×10^7		1.92×10^7		2.10×10^7	
		22	6.61×10^5	96.50	4.21×10^5	97.81	9.12×10^4	99.57
2	"	0	2.00×10^7		2.50×10^7		1.90×10^7	
		22	1.45×10^6	92.80	6.36×10^5	97.46	2.37×10^5	98.77
Control	"	0	1.99×10^7		2.18×10^7		1.90×10^7	
		22	1.52×10^7	23.60	1.69×10^7	22.28	1.44×10^7	24.21
1	Worst Case	0	1.61×10^7		2.03×10^7		1.32×10^7	
		22	1.27×10^6	92.10	1.17×10^5	99.42	4.47×10^4	99.66
2	"	0	2.09×10^7		2.20×10^7		1.45×10^7	
		22	9.35×10^5	95.50	6.12×10^5	97.22	4.60×10^4	99.68
Control	"	0	2.76×10^7		6.96×10^6		1.51×10^7	
		22	1.91×10^7	30.80	4.21×10^6	39.50	5.89×10^6	61.00

Figure 8.

PROTOZOAN SIMULANT (LATEX BEADS) REMOVALS WITH CHLOR-FLOC



* Averages of pH 4.5, 7.0, and 9.0 waters

** Averages of duplicate tests

TABLE 12. REDUCTION OF CRYPTOSPORIDIUM OOCYSTS AND PROTOZOAN SIMULANT WITH ONE CHLOR-FLOC TABLET AT 10 °C WITH pH ADJUSTED TO MAINTAIN 9.0

Water Type	Sample #	Time	Cryptosporidium parvum Oocysts/L	% Reduction	Cyst Simulant Accubeads/L	% Reduction
DF[1]	1	0	1.69×10^6		2.32×10^7	
		22	1.20×10^4	99.29	1.51×10^6	93.49
DF	2	0	1.77×10^6		2.54×10^7	
		22	9.30×10^3	99.47	1.59×10^6	93.74
DF	Control	0	1.77×10^6		2.41×10^7	
		22	1.41×10^6	20.34	1.90×10^7	21.16
Tap	1	0	1.73×10^6		1.45×10^7	
		22	1.10×10^4	99.36	7.12×10^5	95.09
Tap	2	0	1.73×10^6		1.45×10^7	
		22	3.77×10^4	97.80	9.17×10^5	93.70
Tap	Control	0	1.73×10^6		1.45×10^7	
		22	1.80×10^6	-4.05	9.89×10^6	31.79
WC[2]	1	0	1.63×10^6		1.51×10^7	
		22	4.00×10^3	99.75	4.40×10^4	99.71
WC	2	0	1.63×10^6		1.51×10^7	
		22	$<1.00 \times 10^3$	>99.90	6.80×10^4	99.55
WC	Control	0	1.63×10^6		1.51×10^7	
		22	8.00×10^5	50.90	$\times 10^6$	61.00

DF[1]: demand free water
WC[2]: worst case water

2. UA STUDY-PHASE I

a. Median infective dose validation

Initial studies were performed to validate the median infective dose (ID_{50}) for *Cryptosporidium* oocysts in the neonatal mouse model. Two groups of 32 neonatal mice, 5 to 7 days old, were challenged with doses of oocysts which had been exposed to DF water at 5 and 10 °C for 20 minutes. These data showed that the ID_{50} was 67 oocysts in 5 °C water and 80 oocysts for 10 °C water. The combined ID_{50} was 72 oocysts. This combined value was used to determine the experimental and positive control challenge doses because the resulting larger group sizes would provide a more reliable measure of the ID_{50} for these oocyst preparations.

b. Positive controls

Two groups of neonatal mice were challenged with oocysts exposed to each of the test water conditions without disinfection. One group was given the ID_{50} of 72 oocysts (low dose) while the other received 720 oocysts (high dose). Complete data are shown in Table 13. All mice challenged with the high dose showed a high percentage and level (intensity) of infection. Mice challenged with low doses of oocysts exposed to DF water showed the lowest infectivity, while those challenged with oocysts in worst case water showed high infectivity even at the low dose.

TABLE 13. INFECTIVITY OF POSITIVE CONTROLS

Water Treatment	Cyst Dose	Percent Neonatal Mice Infected
5 °C DF*	720	100 (6 of 6)
5 °C DF	72	43 (3 of 7)
5 °C WC	720	100 (6 of 6)
5 °C WC	72	86 (6 of 7)
5 °C WC pH 9.0	720	100 (5 of 5)
5 °C WC pH 9.0	72	100 (7 of 7)
5 °C WC pH 5.0	720	100 (7 of 7)
5 °C WC pH 5.0	72	80 (4 of 5)

* Unless designated otherwise the pH was approximately 7.0.

c. Experimental

The goal of the test was to determine if there was a significant Cryptosporidium oocyst disinfection capability by CHLOR-FLOC when used at recommended concentrations. Desired levels of disinfection were three orders of magnitude (3 $\log_{10}$). For comparison some testing was also conducted on Army issue Globaline (iodine tablets) to determine the relative differences in effectiveness of the two chemicals. Test waters were seeded with 1.0×10^8 oocysts per liter before either CHLOR-FLOC or Globaline tablets were added. After the disinfectant contact period was reached, the chlorine and iodine in the test waters were neutralized; and the oocysts were concentrated as described earlier (recoveries greater than 99 percent by the method described). Microscopic counts of the final sample concentrate were performed using phase microscopy or monoclonal fluorescent tagged antibody (antibody for the cyst wall) counting procedures to quantify cysts for animal dosing. Two groups of neonatal mice were challenged with oocysts exposed to each of the test water disinfectants. One group was given a "low dose" of 7,200 oocysts (2 logs above the ID_{50} of 72 oocysts) while the other received the "high dose" of 72,000 (3 logs above the ID_{50}). According to this dosing protocol if any of the mice challenged with the low dose became infected, then a 3-log reduction in cyst infectivity would not have been achieved. This indeed appears to be the case (Table 14). Except for two CHLOR-FLOC treated groups, involving 5 and 10 °C worst case water, all mice showed unmistakable signs of Cryptosporidium infection. This appears in contradiction to controls when worst case was highly infective.

TABLE 14. INFECTION OF MICE RECEIVING CHLOR-FLOC OR GLOBALINE TREATED OOCYSTS

Water Treatment	Cyst Dose	Neonatal Mice Percent Infected
5 °C DF CHLOR-FLOC*	72,000	100 (14 of 14)
5 °C DF CHLOR-FLOC	7,200	100 (10 of 10)
10 °C DF CHLOR-FLOC	72,000	100 (9 of 9)
10 °C DF CHLOR-FLOC	7,200	100 (11 of 11)
5 °C WC CHLOR-FLOC	72,000	100 (10 of 10)
5 °C WC CHLOR-FLOC	7,200	91 (10 of 11)
5 °C WC CHLOR-FLOC pH 9	72,000	100 (12 of 12)
5 °C WC CHLOR-FLOC pH 9	7,200	100 (12 of 12)
10 °C WC CHLOR-FLOC	72,000	91 (10 of 11)
10 °C WC CHLOR-FLOC	7,200	100 (13 of 13)
5 °C DF I_2	72,000	100 (12 of 12)
5 °C DF I_2	7,200	100 (13 of 13)
5 °C WC I_2	72,000	100 (13 of 13)
5 °C WC I_2	7,200	100 (14 of 14)

* Unless specified the pH during the test was approximately 4.5 to 5.0 due to the buffering capacity of the CHLOR-FLOC and Globaline tablets.

3. UA STUDY-PHASE II

a. Mean infective dose

The median infective dose was determined for the oocyst preparation used in this study by the excystation method discussed in the materials and methods. Excystation was accomplished in triplicate according to the protocol. The percent theoretical sporozoite yield was determined according to the following formula:

$$\text{Percent Theoretical Sporozoite Yield} = \frac{\text{Sporozoites counted}}{4(\text{Intact cysts} + \text{Shells})} \times 100$$

ID_{50} Excystation Data:

Trial	Intact	Shells	Sporozoites
1	56	193	298
2	46	162	284
3	49	158	324
Mean	50.3	171	302

$$\% \text{ Theoretical Sporozoite Yield} = \frac{302}{4(50.3 + 171)} \times 100 = 34.12\%$$

The $\log_{10}$ of this value was used to determine the expected ID_{50} from the chart. The regression line on the chart is based on the log change of sporozoite yield vs. the log change in ID_{50}. Therefore, the regression line equation was used to calculate the ID_{50} (Appendix 2).[8]

b. Positive controls for oocyst recovery efficiency and infectivity

The four test waters (DF, 5 and 10 °C; WC, 5 and 10 °C) were seeded with 1.19×10^8 oocysts per liter. After 20 minutes, the preparations were stirred, and 1 ml was withdrawn for the positive infectivity control challenge doses. The remaining test waters were filtered through 1 µm polycarbonate filters (for DF distilled water) or centrifuged (for WC water) to recover the oocysts. Centrifugation was required for the WC waters due to filter clogging.

DF mean recovery = 71.1 percent
WC mean recovery = 45.0 percent

These values were used to determine the volume of concentrate needed to prepare the challenge doses for each experiment.

Two groups of neonatal BALB/c mice were challenged with oocysts exposed to each of the test waters. One group was given the ID_{50} of 115 oocysts (low test dose) while the other received 1150 oocysts (high test dose). Complete data are shown below

(Table 15). All DF mice challenged with the high dose showed a high level of infection. Mice challenged with WC 5 °C water showed the 50 and 100 percent infection expected for the low and high dose respectively. Mice challenged with the WC 10 °C water did not show this pattern, however. In this case 75 percent of mice challenged with low dose were infected, while only 50 percent of mice challenged with high dose became infected. Since oocysts tend to adhere to and clump with the particles present in worst case water, it is possible that one mouse in each of these groups received less than the expected dose. The outcome of the positive control experiment (Table 14) supported the expected ID_{50} and established the infectivity of the oocysts used in the tests.

TABLE 15. POSITIVE CONTROL DATA

Water	Dose # of oocysts	Treatment	% Infected
DF 5 °C	115	Control	50 (2/4)
DF 5 °C	1150	Control	80 (4/5)
DF 10 °C	115	Control	60 (3/5)
DF 10 °C	1150	Control	100 (5/5)
WC 5 °C	115	Control pH 5	50 (2/4)
WC 5 °C	1150	Control pH 5	100 (2/2)
WC 10 °C	115	Control pH 5	75 (3/4)
WC 10 °C	1150	Control pH 5	50 (1/2)

c. CHLOR-FLOC infectivity test

The results of disinfection/filtration evaluations with CHLOR-FLOC and the associated reaction bag/flannelette filtration procedures to clarify the water of the flocculated materials (including organisms and debris) are shown in Table 16. As in the phase I studies, one tablet was used at temperatures of 10 °C and two tablets were provided at 5 °C. After the CHLOR-FLOC treatment, sample processing, concentration, and oocyst quantification were completed, neonatal mice were infected with the control ID_{50} oocyst dose as well as 100, 1000, and 10,000 ID_{50} dose levels. According to this infectivity protocol, no mice becoming infected would confirm at least a 4-log decrease of infectivity. The data shown below indicate that the experimental treatments caused no measurable reduction in oocyst infectivity. A 99.9 percent (3 logs) reduction in viable oocyst concentration was not achieved. This is supported by the approximately 50 percent infection in mice challenged with the ID_{50} and the almost universal 100 percent infection of mice challenged with doses of oocysts that exceed the ID_{50}.

TABLE 16. CHLOR-FLOC INFECTIVITY DATA FOR PHASE II

Water	Dose # of Oocysts	Treatment	% Infected
DF 5 °C	ID_{50}	2 Tablets	60 (6/10)
DF 5 °C	100 ID_{50}	2 Tablets	100 (10/10)
DF 5 °C	1000 ID_{50}	2 Tablets	100 (10/10)
DF 5 °C	10000 ID_{50}	2 Tablets	100 (10/10)
DF 10 °C	ID_{50}	1 Tablet	60 (6/10)
DF 10 °C	100 ID_{50}	1 Tablet	80 (8/10)
DF 10 °C	1000 ID_{50}	1 Tablet	100 (9/9)
DF 10 °C	10000 ID_{50}	1 Tablet	100 (10/10)
WC 5 °C	ID_{50}	2 Tablets pH 5	100 (10/10)
WC 5 °C	100 ID_{50}	2 Tablets pH 5	100 (10/10)
WC 5 °C	1000 ID_{50}	2 Tablets pH 5	100 (10/10)
WC 5 °C	10000 ID_{50}	2 Tablets pH 5	100 (10/10)
WC 10 °C	ID_{50}	1 Tablet pH 5	100 (10/10)
WC 10 °C	100 ID_{50}	1 Tablet pH 5	100 (10/10)
WC 10 °C	1000 ID_{50}	1 Tablet pH 5	100 (10/10)
WC 10 °C	10000 ID_{50}	1 Tablet pH 5	100 (10/10)
Tap 5 °C	100 ID_{50}	1 Tablet	60 (3/5)
Tap 5 °C	1000 ID_{50}	1 Tablet	100 (10/10)

d. Alternative viability test of CHLOR-FLOC treated oocysts

A small portion of the final concentrated sample after CHLOR-FLOC treatment was evaluated for viability of the residual oocysts using monoclonal antibody. The physical oocyst removal by the CHLOR-FLOC system included the settling and removal of the floc that was formed by the coagulant component contained in CHLOR-FLOC with the particulates contained in the challenge water using the flannelette filters. The viability of the oocysts was determined using a monoclonal antibody, OW 64 MAb, that was developed by Dr. Sterling's laboratory at the UA Department of Veterinary Science to determine the degradation or partial opening of cyst suture lines. Previous studies by that laboratory[9] have provided a linear regression analysis establishing a correlation between binding of the OW 64 MAb monoclonal antibody to the cyst suture and loss of cyst infectivity. Table 17 shows only a small portion of OW 64 positive oocysts found after CHLOR-FLOC treatment, thus indicating that the disinfectant treatment was not effective in reducing the viability of the oocysts. This finding supports the results of the neonatal mouse infectivity studies. The results also show that only a very small component of the cyst challenge

was physically removed by the coagulation, sedimentation, and filtration processes of the CHLOR-FLOC treatment. It was observed in the trials that the CHLOR-FLOC sediment had a tendency to hang up on the sides of the plastic bag and was not readily settled, thus impacting on the amount of material that could be wasted before filtration through the flannelette bag. The bags obviously provided little clarification of the residual floc as can be seen in Figure 4c.

TABLE 17. RECOVERY AND VIABILITY OF CHLOR-FLOC TREATED OOCYSTS

Treatment	Oocyst Challenge/L	Oocysts Recovered/L	% Oocysts Recovered	% Oocysts OW64 Positive
DF 5 °C	1.0×10^8	3.72×10^7	37.2	0.42
DF 10 °C	1.0×10^8	3.32×10^7	33.2	0.0
WC 5 °C	1.0×10^8	1.90×10^7	19.2	0.25
WC 10 °C	1.0×10^8	3.81×10^7	38.1	0.14

DISCUSSION

1. USABRDL IN-HOUSE STUDIES

The results of in-house studies indicated that the CHLOR-FLOC tablets were very effective at temperatures of 5 °C, using 2 tablets, and 10 °C, using 1 tablet, for the destruction of both the Klebsiella terrigena and Echovirus 1; achieving over a 10^6 reduction in the bacterial challenge and over 10^4 reduction in the virus challenge. These removals were achieved within the first 5 minutes of contact even before the materials were filtered through the flannelette materials at 20 minutes. The pH of the initial waters before CHLOR-FLOC addition did not appear to have a significant impact on the effectiveness of the disinfectant; however, in all cases the initial pHs, even as high as pH 9.0, were quickly reduced to less than pH 5.0 due to the low pH buffering capacity of the tablets. The artificial maintenance of pH 9.0 did not hamper the ability of either one or two tablets to provide the required removals of the bacteria and viruses still within the 5-minute contact period. This was somewhat surprising because higher pH typically reduce the effectiveness of chlorine based-disinfectants. The residual chlorine levels even using one tablet remained in the range of 6-10 mg/liter at the 5-minute sampling time and and 5-9 mg/liter after 20 minutes, regardless of the water quality challenge (including worst case water). Control tests showed that the flannelette material had no significant removal potential for the organisms; but because of the activity of the disinfectant component, the effectiveness of the bags in the presence of the coagulant component alone of the CHLOR-FLOC system was not evaluated.

The above results are somewhat different than observed for the bacteria used in studies by Powers.[10] He did not detect any residual bacteria in natural waters dosed with E. coli or Pseudomonas when disinfected with either CHLOR-FLOC or iodine tablets over a 10-minute contact time. However, in further studies[11] he indicated that even after 20 minutes there were a few residual E. coli survivors in the flocculated material. These organisms were not found in the water, whereas we had total destruction of Klebsiella within 5 minutes in the water and floc. It cannot be ascertained if the E. coli were more resistant or somehow became entrained in the flocculated material, thus contributing to their survival. Additionally, studies by Dugway Proving Ground[12] on Poliovirus 1 (Chat strain) and simian rotavirus (SA-11 Strain) indicated that virus disinfection was not entirely adequate. In their study, using the EPA guide standard and protocol, they attained over the required 4-log removal of rotavirus, but only achieved several logs removal of Poliovirus 1. Poliovirus removals ranged from 2.5 to 2.7 logs at 5 °C using two CHLOR-FLOC tablets within 40 minutes. Companion studies with two iodine tablets showed only 1.0 to 2.2 log

removals in a similar time period. It cannot be determined why there was such a discrepancy in virus disinfection between our efforts and those of the Dugway Proving Ground. It is possible that the strain of poliovirus they were using was more resistant to the CHLOR-FLOC than the echovirus strain of our Laboratory. Also, it is not known what procedure they used for preparing their stock virus; possibly, their virus preparation had a high ratio of large virus clumps, which is known to change the disinfection kinetics of similar enteroviruses due to the protective effects of the outer virions for the inner ones of the aggregates.

The physical removal of the Cryptosporidium parvum oocysts by the CHLOR-FLOC system indicated less than required removals of 10^3 cysts. This was also noted for the latex beads (simulant of cyst particles). It was apparent that the coagulant/filtration component did not work effectively in providing either adequate size flocs or small enough filter matrix to trap the cyst and simulant particles. As would be expected, however, the worse the challenge water quality, the better the floc formation and the better the cyst-size particle removal. Similarly, the increased temperature experiments showed slightly improved removals probably because of improved flocculation. Maintenance of pH 9.0 did allow the removals to approach the 10^3 target for both cysts and simulant. The relative removal of the simulant latex beads was very comparable to that for oocysts under all of the challenge conditions and appeared to be a good model for cyst removal.

2. UA PHASE I STUDY

Cryptosporidium infection was noted in all groups of neonatal mice given oral doses of oocysts treated by either CHLOR-FLOC (not filtered through flannelette material) or Globaline water disinfectants. The results demonstrated that the activity of these chemicals used to treat the Cryptosporidium-seeded test waters did not achieve 3-log reductions in oocyst viabilities. The high level of infection observed in the groups challenged with the low dose also may allow the ruling out of a 2-log reduction. There may have been a reduction of less than 2 logs of infective oocysts with CHLOR-FLOC, but the challenge dose levels used in the tests tend to mask any reduction of lower magnitude. During microscopic examination, a few experimental histology specimens were observed to have lower levels of infection than others. This phenomenon was not seen in any of the control specimens which all showed heavy infection. Reduced levels of cysts in the animal specimens may have indicated some level of degradation in the cyst integrity by the CHLOR-FLOC, thus leading to reduced levels of infection in the animal host. Infectivity studies with the Globaline disinfectant containing iodine indicated no susceptibility of the oocysts to the disinfectant at either low or high doses. Also, there was no

indication of reduced infectivity level in histological samples for those mice fed Globaline-treated oocysts. The results would indicate that neither CHLOR-FLOC nor Globaline were effective disinfectants for Cryptosporidium oocysts, and that neither could approach a 3-log reduction in infectivity as required in the USEPA's "Guide Standard and Protocol for Testing Microbiological Water Purifiers."

3. UA PHASE II STUDY

The results of the phase II tests revalidated the very minimal capabilities of CHLOR-FLOC to disinfect Cryptosporidium oocysts in either distilled water, tapwater, or worst case water at 5 and 10 °C. Also the parallel tests of infectivity using the OW64 MAb monoclonal antibody against the oocyst suture (which show up after loss of viability of the cysts) indicated a very low percentage of oocysts positive for this suture directed antibody. The results also indicated that physical removal of oocysts by the coagulation-flocculation process and filtration through flannelette material had little capability to physically remove oocysts. The experimental CHLOR-FLOC reaction bags tended to not allow proper settling of the floc that formed by coagulation; thus, the bulk of this material containing oocysts could not properly be voided prior to the filtration step.

The overall study results revealed that the CHLOR-FLOC system, as presently configured, was not adequate to physically remove, or provide adequate chemical disinfection of, Cryptosporidium oocysts to the required levels of 99.9 percent reduction recommended by the USEPA's "Guide Standard and Protocol for Testing Microbiological Water Purifiers." Improved removal of floc and filtration would significantly enhance the ability of the CHLOR-FLOC to physically remove protozoan cysts and thus help meet the criteria. The disinfection of oocysts by iodine was negligible, thus indicating that some sort of filtration or other procedure would be necessary to ensure that the oocysts were physically removed before consumption.

CONCLUSIONS

1. The results support the efficacy of major components of the USEPA guide standard and protocol for the evaluation of the disinfection/removal effectiveness of CHLOR-FLOC.

2. CHLOR-FLOC will provide adequate removals of typical waterborne test indicator bacteria and the enteroviruses at low temperatures, even when water is maintained at high pH.

3. The bacterial and virus removal results support the USEPA's acceptance of this chemical disinfectant/coagulant mixture for recreational and emergency disinfection of water.

4. While no direct comparison of CHLOR-FLOC against calcium hypochlorite or iodine tablet disinfection was made, results from previous contract studies[13] for iodine disinfection indicate CHLOR-FLOC provides at least equal or better results for viruses.

5. The physical removal of Cryptosporidium parvum cysts and 3.7 μm AccuBead™ cyst simulant did not continuously meet the reductions required by the USEPA guide standard.

6. If it were necessary for the Army to use CHLOR-FLOC at this time, improvements in the effectiveness of water filtration after CHLOR-FLOC treatment (such as filtration through 3.0 μm absolute rated pore size filters) would provide the increased cyst removal capabilities needed to meet field use considerations.

Appendix 1

EXCYSTATION OF CRYPTOSPORIDIUM OOCYSTS

Materials: Freshly washed oocysts (Pelleted in a 15 ml conical tube)

tissue Culture PBS (TPBS)

2× Excystation Medium (Frozen medium must be allowed to reach room temperature before use.)

Procedure: (Note: This procedure may be adapted for use with very small volumes. In this case, use 1.5 ml Eppendorf tubes, wash with 1 ml TPBS, centrifuge at setting #7, aspirate down to 100 µl, and add 100 µl of 2× excystation medium.

1. Carefully add 5 ml of TPBS to the freshly washed oocysts without disturbing the pellet. Centrifuge at 3000 rpm for 3-5 minutes. Repeat this step once. (Note: centrifuge at 3000 rpm for 10 minutes if the pellet was disturbed.)

2. Aspirate down to 0.5 ml, trichurate with a pasteur pipette to break up the pellet, and bring the volume to 1.0 ml with 2× excystation medium. Place in 37° C water bath for 60 minutes. Remove from the water bath and let the sample sit at room temperature for 30 minutes before counting. Place on ice if counting cannot proceed immediately.

3. Count and record at least 200 intact cysts + shells and the sporozoites in the same area of the counting chamber. Repeat this once using another sample from the excystation mixture.

4. To calculate the percent excystation, divide the number of shells by the sum of intact cysts and shells and multiply by 100. Calculate the number of sporozoites produced per shell by dividing the number of sporozoites by the number of shells.

$$\text{Percent Excystation} = \frac{\text{Shells}}{\text{Intact Cysts + Shells}} \times 100$$

$$\text{Sporozoites per Shell} = \frac{\text{Sporozoites}}{\text{Shells}}$$

4. Calculate the percent theoretical sporozoite yield by dividing the number of sporozoites observed by 4× the sum of the number of shells and the number of intact cysts.

$$\text{Percent Theoretical Sporozoite Yield} = \frac{\text{Sporozoites}}{4(\text{Intact Cysts + Shells})} \times 100$$

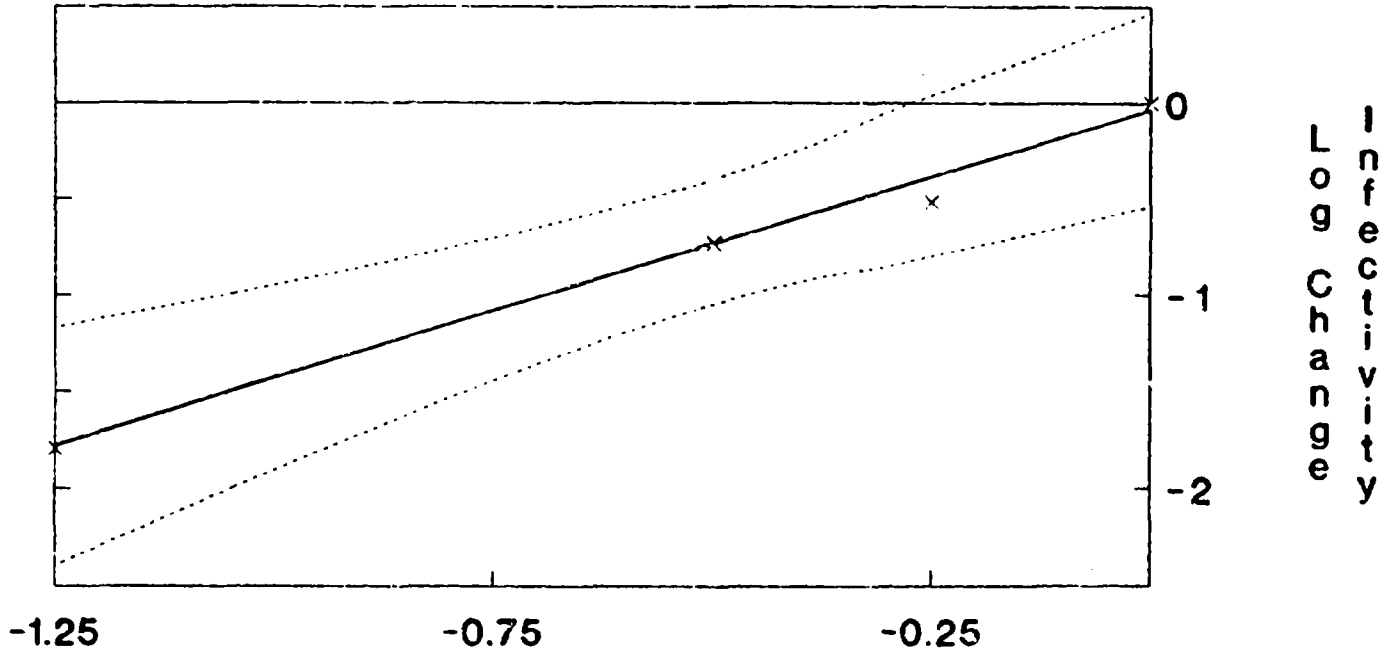
Ref: Woodmansee. 1987. J. Protozool. **34(4)**:398-402.

Appendix 2

Infectivity v Sporozoite Yield

Linear Regression Analysis

80% Probability to Predict Viability with One Trial



Regression line: y = 1.39(x) - 0.066
r = 0.995, P<0.0048

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